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Synthesis and vasodilatory activity of new *N*-acylhydrazone derivatives, designed as LASSBio-294 analogues

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Abstract—Conventional therapy to treat hypertension often involves arterial vasodilation. Decrease of blood pressure by vasodilators is normally associated with adverse effects because of their low vascular selectivity. This is of interest to develop new molecules with potential for clinical use and fewer side effects. Recently, a new bioactive compound of the *N*-acylhydrazone class, LASSBio-294, was shown to produce a cardioinotropic effect and vasodilation. In this report, new derivatives of LASSBio-294 were designed and tested on the contractile response of vascular smooth muscle from Wistar rats. Phenylephrine-induced contracture in the aorta was inhibited by the derivatives LASSBio-785 and LASSBio-788. The concentrations necessary to cause 50% reduction of the maximal vascular response (IC_{50}) were 10.2 ± 0.5 and 67.9 ± 6.5 μM. Vasodilation induced by both derivatives is likely to be mediated by a direct effect on smooth muscle because it was not dependent on the integrity of vascular endothelium. LASSBio-785 was seven times more potent than the reference compound LASSBio-294 (IC_{50} = 74 μM) in producing an endothelium-independent vasodilator effect.

1. Introduction

Relaxation of vascular smooth muscle is the basis for the treatment of hypertension.[1] Several pharmacological agents have been synthesized but none of them has had a specific action, free of side effects. It is important to find new vasodilators with a potential for clinical use and not associated with adverse effects.

Recently, new bioactive compounds of the *N*-acylhydrazone class were synthesized from safrole (**1**), a Brazilian natural product obtained from sassafras oil (*Ocotea pretiosa*).[2,3] Replacing the phenyl ring attached to the imine moiety by the isosteric 2-thienyl ring[4] resulted in the design of 3,4-methylenedioxybenzoyl-2-thienylhydrazone, named LASSBio-294 (Chart 1). LASSBio-294 was described as a potent positive cardiac inotropic agent, whose activity was related to increased Ca^{2+} accumulation in the sarcoplasmic reticulum (SR).[5] LASSBio-294 also promoted vasodilation in aortic rings, mediated by the guanylate cyclase/cyclic guanylate monophosphate pathway.[6] Considering that the *N*-acylhydrazone subunit present in LASSBio-294 has a bioisoteric relationship with the pyridazinone ring shared by phosphodiesterase (PDE) inhibitors,[7] the bioprofiles of several synthetic analogues of lead-compound LASSBio-294 were investigated in vascular smooth muscle in an effort to identify new drug candidates with vasodilatory properties and fewer side effects.

The design of the new functionalized LASSBio-294 derivatives, that is, LASSBio-785 to LASSBio-791, explored the change in the electronic density of the pharmacophoric thienyl subunit by the introduction of functional groups (–CH_3, –Br, –NO_2) with different electronic properties in the C-5 of the heterocyclic ring. Additionally, modifications in the stereoelectronic behavior of the acylhydrazone group were performed

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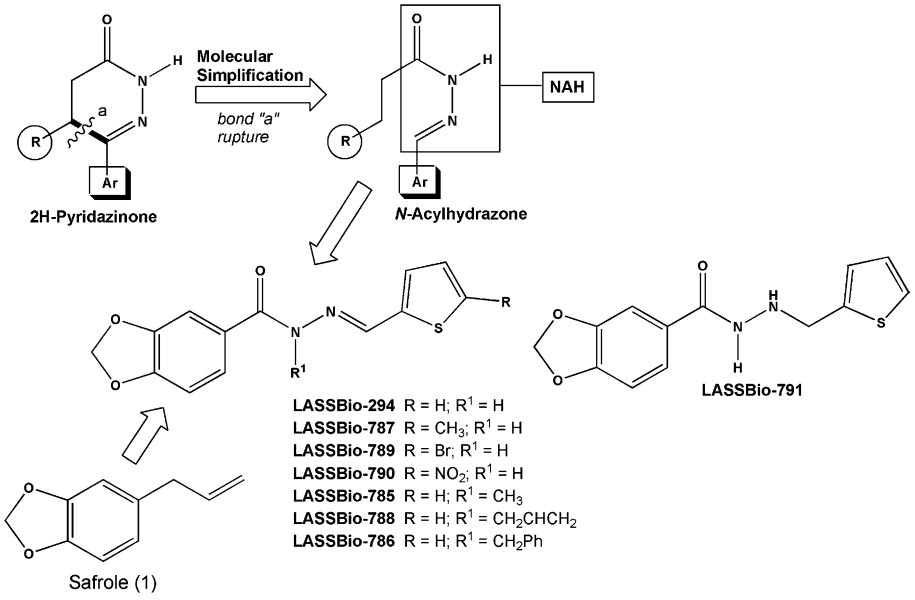


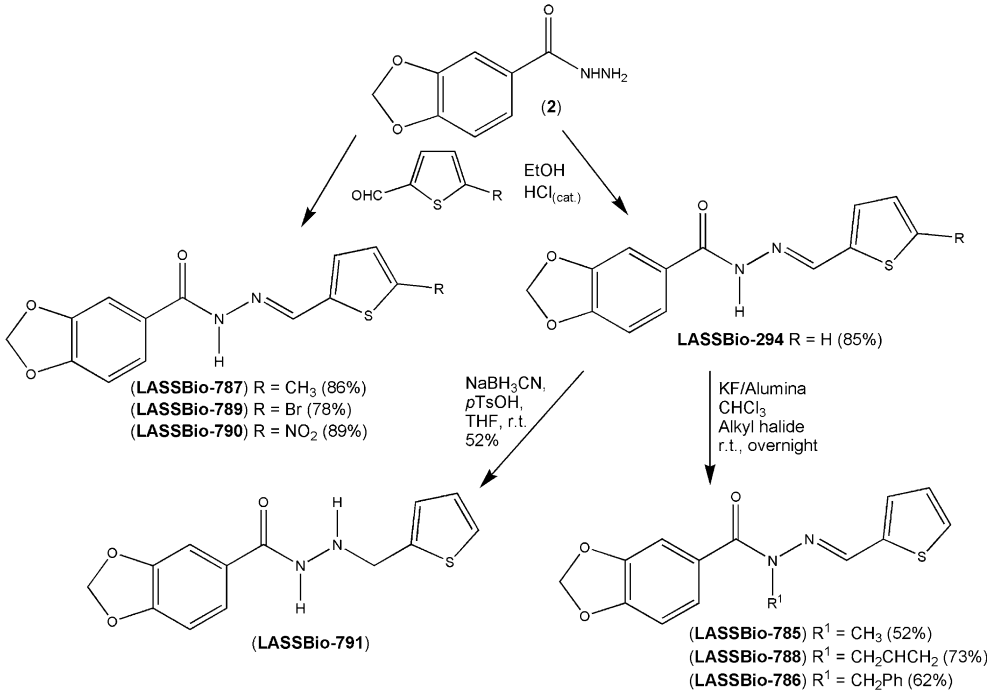
Chart 1. Structural pattern of LASSBio-294 and its new functionalized analogues.

through its *N*-alkylation with methyl, allyl, and benzyl groups or by the selective reduction of its imine function (Chart 1).

2. Chemistry

The synthesis of the new 3,4-methylenedioxybenzoyl hydrazone derivatives LASSBio-785 to LASSBio-790 is outlined in Scheme 1. The starting material was the 3,4-methylenedioxybenzoyl hydrazine (**2**) obtained in 57% overall yield from natural safrole (**1**), as described previously.[3] The initial step consisted in the acid-catalyzed condensation[3] of the hydrazine (**2**) with 2-thiophenecarboxaldehyde or its C-5 functionalized derivatives with methyl, bromine or nitro groups,[8] which furnished the LASSBio-294 derivative and the desired acylhydrazone derivatives LASSBio-787, LASSBio-789, and LASSBio-790 in very good yields (Scheme 1).

Next, compound LASSBio-294 was employed as precursor of the *N*-alkylated derivatives LASSBio-785, LASSBio-786, and LASSBio-788, through its treatment with



Scheme 1. Synthetic route exploited in the preparation of the target compounds LASSBio-785 to LASSBio-791.

alumina supported potassium fluoride in chloroform, followed by the addition of the appropriate alkyl halides at room temperature.[9] By applying this procedure we were able to obtain, selectively, the *N*-methyl (LASSBio-785), *N*-benzyl (LASSBio-786), and *N*-allyl (LASSBio-788) in yields ranging from 52% to 73% after purification by recrystallization or silica gel column chromatography (Scheme 1).

Finally, the reduced derivative LASSBio-791 could be obtained in 52% yield from acid-catalyzed chemoselective reduction of the imine group with sodium cyanoborohydride in THF (Scheme 1).[10]

The careful analysis of ^{1}H NMR spectra of the new acylhydrazone derivatives described herein, allowed us to detect the presence of only one imino hydrogen signal. This was attributed to the (*E*)-diastereomer on the basis of several previous reports from our group describing the configuration of bioactive acylhydrazone compounds.[2,3,11–14] Additionally, analytical results for C, H, and N for all seven new target compounds were within ±0.4% of the theoretical values.

3. Results and discussion

Vasodilation activity was investigated in aortic rings pre-contracted with 10 μM phenylephrine. Figure 1A shows a typical recording of the maximal contractile response of aorta induced by phenylephrine followed by exposure to acetylcholine (10 μM). The relaxation observed in the presence of acetylcholine indicates that the endothelium was intact in that aorta. Thirty minutes after washout, the preparation was exposed to increasing concentrations of LASSBio-785 after the plateau phase of phenylephrine-induced contracture. LASSBio-785 promoted relaxation in a dose-dependent manner with the maximal effect observed at 100 μM. Other compounds were less effective (Fig. 1B). Among all compounds tested, LASSBio-785 and LASSBio-788 were the more potent in reducing the phenylephrine-induced contracture in aortic rings. At 200 μM, the amplitude of contracture was significantly reduced to 4.4 ± 2.8% ($n = 6$) and 10.4 ± 3.2% ($n = 6$) of control by LASSBio-785 and LASSBio-788, respectively. The concentrations necessary to reduce by 50% the maximal contraction induced by phenylephrine (IC_{50}) in aorta

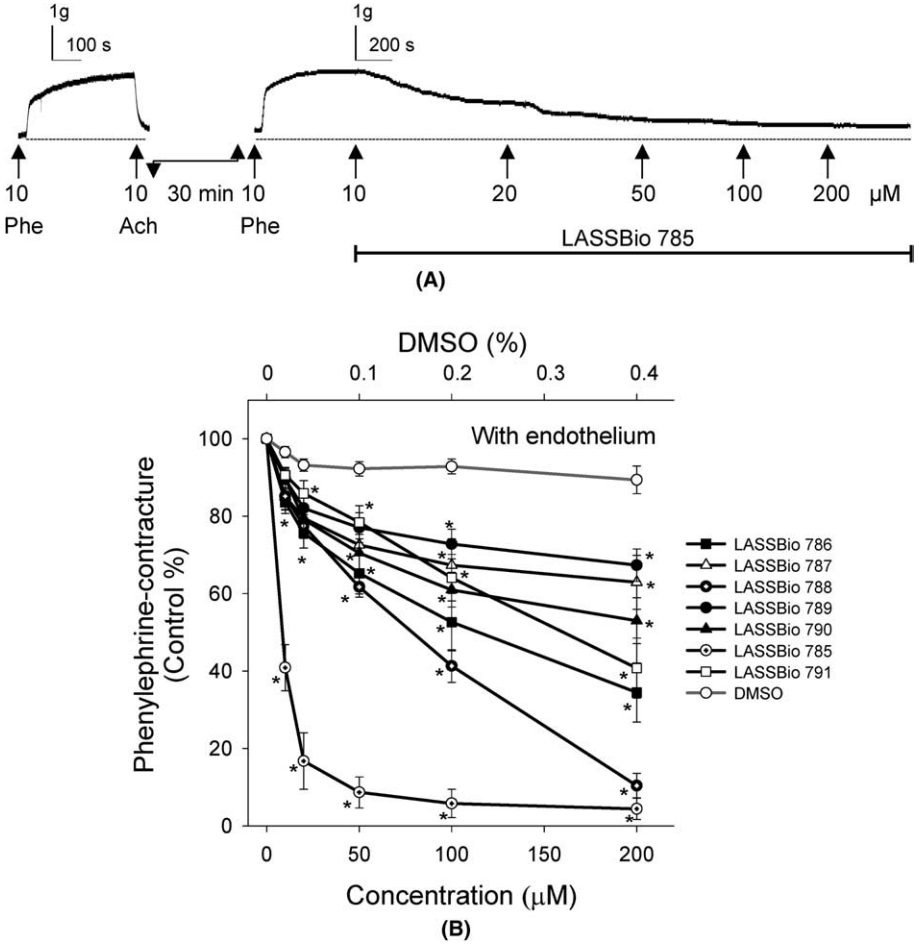


Figure 1. Effects of derivatives on contracture induced by phenylephrine (Phe) in rat aortic rings. (A) Representative tracing of tension in response to 10 μM Phe followed by exposure to 10 μM acetylcholine (Ach). After 30 min washout, phenylephrine-induced contracture was observed in the presence of increasing concentrations of LASSBio-785 (10–200 μM). (B) Concentration–response curves for derivatives in aorta with intact endothelium. Data represent mean ± SEM of 8 experiments. $^*P < 0.05$, compared with control value.

Table 1. The concentration of compounds necessary to reduce maximal phenylephrine-induced contracture by 50% (IC_{50}) in aorta with or without intact endothelium

Compounds	IC_{50} (μM)	
	With endothelium	Without endothelium
LASSBio-785	10.2 ± 0.5	18.5 ± 3.6
LASSBio-788	67.9 ± 6.5	65.7 ± 8.0
LASSBio-786	134.1 ± 31.0	141.6 ± 53.8
LASSBio-791	172.8 ± 26.7	ND
LASSBio-790	216.0 ± 39.3	ND
LASSBio-787	293.0 ± 76.0	273.4 ± 22.0
LASSBio-789	ND	ND
LASSBio-294	74.0	ND

ND = Not determined.

with intact endothelium were 10.2 ± 0.5 and 67.9 6.5 μM (Table 1). The IC_{50} of LASSBio-785 was seven times lower than LASSBio-788 and almost thirty times lower than LASSBio-787 (293.0 ± 76.0 μM). LASSBio-787, LASSBio-789, LASSBio-790, and LASSBio-791 (200 μM) significantly reduced the phenylephrine-induced contracture to 37.1 ± 7.0% ($n = 6$), 32.7 ± 4.2 ($n = 6$), 47.0 ± 5.9% ($n = 6$), and 59.2 ± 7.8% of control, respectively.

The importance of endothelial integrity to the vasodilatory action of new derivatives was evaluated in experiments in which those cells were mechanically removed from the aorta. Removal of endothelium was confirmed by the lack of relaxation in the presence of acetylcholine, as shown in Figure 2A. Note that only a slight vascular relaxation was observed at a high concentration of LASSBio-790 (200 μM). This indicates that vasodilation induced by LASSBio-790 was partially dependent on the presence of intact endothelium (Fig. 2B). Compared to aorta having intact endothelium, no significant difference was observed in the dose–inhibitory response curve for endothelium-free aorta in the presence of LASSBio-785 (Fig. 2B). The IC_{50} for LASSBio-785 in aorta with intact endothelium was not significantly different from aorta without endothelium (18.5 ± 3.6 μM, $P = 0.8$) (Table 1). Thus, mechanical removal of the endothelium did not alter the IC_{50} values of LASSBio-785 for

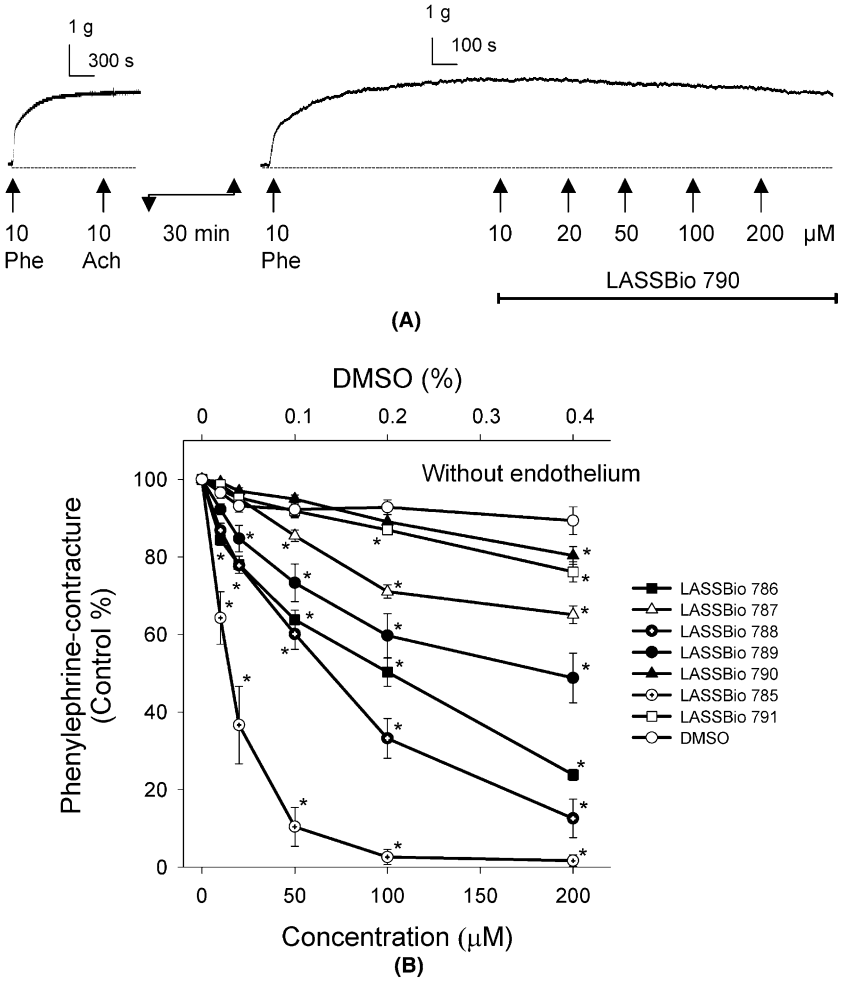


Figure 2. Effects of derivatives on contracture induced by phenylephrine (Phe) in rat aortic rings without endothelium. (A) Representative tracing of tension in response to 10 μM Phe followed by exposure to 10 μM acetylcholine (Ach). Note lack of relaxation after treatment with Ach confirming the removal of endothelium. After 30 min washout, phenylephrine-induced contracture was observed in the presence of increasing concentrations of LASSBio-790 (10–200 μM). (B) Concentration–response curves for derivatives in aorta without intact endothelium. Data represent mean ± SEM of eight experiments. $^{*}P < 0.05$, compared with control value.

inhibiting the phenylephrine-induced contracture in aortic rings. Vascular relaxation was totally reversed after 30 min washout.

These results indicate the important contribution of the presence of a small lipophilic group substituting the amide group of the *N*-acylhydrazone moiety to the selective potentialization of the vasodilator properties of LASSBio-785, in detriment of its cardiotonic profile, probably due to the change in side-chain conformation. On the other hand, the introduction of bulky *N*-alkyl substituents or groups with different electronic contributions surrounding the pharmacophoric 2-thienyl ring was not able to improve the vasodilator profile. Finally, the reduced activity displayed by compound LASSBio-791 confirmed the pharmacophoric character anticipated for the *N*-acylhydrazone framework.

4. Conclusion

LASSBio-785 and LASSBio-788 were more potent than other compounds to induce vasodilation in pre-contracted aortic rings. Both derivatives differ from LASSBio-294 in the amidic nitrogen of the *N*-acylhydrazone subunit, in which were introduced methyl and allyl substituents, respectively. LASSBio-788 was equipotent to LASSBio-294, as its IC_{50} was similar (72.5 ± 8.6 μM) to that previously described for LASSBio-294, 74 μM.[7] A significant change in pharmacological profile was observed with LASSBio-785, where IC_{50} was 10.2 ± 0.5 μM, seven times higher than for LASSBio-294. Vascular relaxation observed in the presence of some compounds was not affected by endothelium removal. Lack of functional endothelium only altered the activity for LASSBio-790 and LASSBio-791. These results indicate that vascular relaxation is a consequence of a direct action on the vascular smooth muscle.

5. Experimental

5.1. Chemistry

Melting points were determined with a Quimis 340 apparatus and are uncorrected. ^{1}H NMR spectra were determined in deuterated chloroform containing ca. 1% tetramethylsilane as an internal standard, using a Varian Gemini 300 at 300 MHz. ^{13}C NMR spectra were determined in the same spectrometer at 75 MHz, employing the same solvents. Microanalyses were carried out using a Perkin Elmer 240 analyzer and Perkin Elmer AD-4 balance.

The progress of all reactions was monitored by thin-layer chromatograph, which was performed on 2.0×6.0 cm aluminum sheets precoated with silica gel 60 (HF-254, Merck) to a thickness of 0.25 mm. The developed chromatograms were viewed under ultraviolet light (254–265 nm) and treated with iodine vapor. For column chromatography, Merck silica gel (70–230 mesh) was used. Reagents and solvents were purchased from commercial suppliers and used as received. The usual work-up means that the organic extracts, prior to concentration under reduced pressure, were treated with a saturated aqueous sodium chloride solution, referred as to brine, dried over anhydrous sodium sulfate and filtered.

5.1.1. General procedure for the preparation of 3,4-methylenedioxybenzoyl-acylhydrazones LASSBio-294, LASSBio-787, LASSBio-789, and LASSBio-790. To a solution of 3,4-methylenedioxybenzoylhydrazine (**2**) (0.150 g, 0.83 mmol) in absolute EtOH (7 mL) containing two drops of 37% hydrochloric acid, was added 0.87 mmol of the corresponding thiophene-2-carboxyaldehyde derivative.[8] The mixture was stirred at room temperature for 30 min, until extensive precipitation was visualized. Next, the solvent was partially concentrated at reduced pressure and the resulting mixture was poured into cold water. After neutralization with 10% aqueous sodium bicarbonate solution, the precipitate formed was filtered out and dried under vacuum to give the desired acylhydrazone derivatives LASSBio-294, LASSBio-787, LASSBio-789, and LASSBio-790, as described next.

5.1.2. (2-Thienylidene) 3,4-methylenedioxybenzoylhydrazine (LASSBio-294). The derivative LASSBio-294 was obtained as a yellow solid by condensation of (**2**) with 2-thiophenecarboxyaldehyde[9] in 85% yield, mp 204–205 °C. ^{1}H NMR (200 MHz): δ 11.56 (s, 1H, CON*H*–), 8.62 (s, 1H, =C*H*), 7.64 (d, 1H, H_2', $J = 5.03$ Hz), 7.49 (dd, 1H, H_6, *J*ax = 8.1 Hz, *J*bx = 1.8 Hz), 7.44–7.41 (m, 2H, H_4', H_2), 7.12 (dd, 1H, H_3', *J*ax = 5.04 Hz, *J*bx = 5.03 Hz), 7.03 (d, 1H, H_2', $J = 8.1$ Hz), 6.18 (s, 2H, O–CH_2–O).

5.1.3. (5-Methyl-2-thienylidene) 3,4-methylenedioxybenzoylhydrazine (LASSBio-785). The derivative LASSBio-785 was obtained as a brown solid by condensation of (2) with 5-methyl-2-thiophenecarboxyaldehyde[8] in 86% yield, mp 231–232 °C. ^{1}H NMR (200 MHz): δ 11.56 (s, 1H, CON*H*–), 8.53 (s, 1H, =C*H*), 7.47 (dd, 1H, H_6, *J*ax = 8.2 Hz, *J*bx = 1.7 Hz), 7.40 (d, 1H, H_2, $J = 1.74$ Hz), 7.23 (d, 1H, H_4', $J = 3.6$ Hz), 7.03 (d, 1H, H_5, $J = 8.2$ Hz), 6.82 (d, 1H, H_3', $J = 3.57$ Hz) 6.11 (s, 2H, O–CH_2–O), 2.43 (s, 3H, Ar–CH_3).

5.1.4. (5-Bromo-2-thienylidene) 3,4-methylenedioxybezoylhydrazine (LASSBio-789). The derivative LASSBio-789 was obtained as a beige solid by condensation of (**2**) with 5-bromo-2-thiophenecarboxyaldehyde[9] in 78% yield, mp 201–202 °C. ^{1}H NMR (200 MHz): δ 11.56 (s, 1H, CON*H*–), 8.55 (s, 1H, =C*H*), 7.47 (dd, 1H, H_6, *J*ax = 8.1 Hz, *J*bx = 1.6 Hz), 7.40 (d, 1H, H_2, $J = 1.6$ Hz), 7.30 (d, 1H, H_4', $J = 3.8$ Hz), 7.25 (d, 1H, H_3', $J = 3.84$ Hz), 7.04 (d, 1H, H_5, $J = 8.1$ Hz), 6.07 (s, 2H, O–CH_2–O).

5.1.5. (5-Nitro-2-thienylidene) 3,4-methylenedioxybenzoylhydrazine (LASSBio-790). The derivative LASSBio-790 was obtained as a yellow solid by condensation of (**2**) with 5-nitro-2-thiophenecarboxyaldehyde[8] in 89% yield, mp 234–235 °C. ^{1}H NMR (200 MHz): δ 11.95 (s, 1H,

CON*H*–), 8.81 (s, 1H, =C*H*), 8.1 (m, 2H, H_4', H_3'), 7.56–7.43 (m, 3H, H_2, H_5, H_6), 7.05 (d, 1H, J = 8.1 Hz), 6.07 (s, 2H, O–CH_2–O).

5.1.6. General procedure for selective *N*-alkylation of the compound LASSBio-294. Compound LASSBio-294 (0.5 g) and potassium fluoride supported on alumina[9] (10.0 g) in CH_3Cl (5 mL) were thoroughly mixed under vigorous stirring. Then, methyl iodide, benzyl bromide or allyl bromide (1.2 equiv) was added to the mixture. The reaction was kept overnight with occasional shaking. Next, the reaction was diluted with CH_2Cl_2 (50 mL), filtered, and concentrated. *N*-Methyl (LASSBio-785) and *N*-benzyl (LASSBio-786) derivatives were obtained as a solid, after recrystallization in EtOH. On the other hand, the allyl derivative (LASSBio-788) was obtained as an oil, after silica gel column chromatography (hexane–EtOAc, 9:1).

5.1.7. *N*-Methyl (2-thienylidene) 3,4-methylenedioxybenzoylhydrazine (LASSBio-785). The derivative LASSBio-785 was obtained as a white solid by the *N*-alkylation of LASSBio-294 with methyl iodide, in 52% yield, mp 142–143 °C. ^{1}H NMR (200 MHz): δ 7.92 (s, 1H, =C*H*), 7.40 (dd, 1H, H_6, Jax = 8.2 Hz, Jbx = 1.7 Hz), 7.33–7.22 (m, 3H, H_2, H_4', H_2'), 7.03 (dd, 1H, H_3', Jax = 5.0 Hz, Jbx = 3.7 Hz), 6.85 (d, 1H, H_5, J = 8.2 Hz), 6.05 (s, 2H, O–CH_2–O), 3.57 (s, 1H, N–CH_3).

5.1.8. *N*-Benzyl (2-thienylidene) 3,4-methylenedioxybenzoylhydrazine (LASSBio-786). The derivative LASSBio-786 was obtained as a brown solid by the *N*-alkylation of LASSBio-294 with benzyl bromide, in 62% yield, mp 130–132 °C. ^{1}H NMR (200 MHz): δ 7.83 (s, 1H, =C*H*), 7.50 (dd, 1H, H_6, Jax = 8.2 Hz, Jbx = 1.7 Hz), 7.42 (d, 1H, H_5, J = 1.7 Hz), 7.40–7.24 (m, H_2', CH benzyl), 7.06 (d, 1H, H_4', Jax = 3.5 Hz), 6.96 (dd, 1H, H_3', Jax = 5.0 Hz, Jbx = 3.7 Hz), 6.89 (d, 1H, H_2, J = 8.2 Hz), 6.05 (s, 2H, O–CH_2–O), 5.04 (s, 2H, N–CH_2–benzyl).

5.1.9. *N*-Allyl (2-thienylidene) 3,4-methylenedioxybenzoylhydrazine (LASSBio-788). The derivative LASSBio-788 was obtained as a yellow oil by the *N*-alkylation of LASSBio-294 with allyl bromide, in 73% yield. ^{1}H NMR (200 MHz): δ 7.89 (s, 1H, =C*H*), 7.43 (dd, 1H, H_6, Jax = 8.2 Hz, Jbx = 1.7 Hz), 7.36 (d, 1H, H_2, J = 1.7 Hz), 7.19 (d, 1H, H_2', J = 3.7 Hz), 7.02 (dd, 1H, H_3', Jax = 5.0 Hz, Jbx = 3.7 Hz), 6.86 (d, 1H, H_5, J = 8.2 Hz), 6.03 (s, 2H, O–CH_2–O), 5.98–5.79 (m, 1H, N–CH_2–C*H*), 5.29–5.16 (m, 2H, CH=CH_2), 4.80–4.76 (m, 2H, N–CH_2–CH).

5.1.10. *N*′-(2-Thiophen-2-ylmethyl) 3,4-methylenedioxybenzoylhydrazine (LASSBio-791). In a vessel with nitrogen atmosphere, containing $NaBH_3CN$ (1.776 g, 24 mmol), 3 mmol of LASSBio-294 derivative and bromcresol green (1–2 mg) dissolved in 3.0 mL THF, was added a solution of *p*-toluenesulfonic acid monohydrate (0.576 g, 3 mmol) in THF (3.0 mL), slowly, under vigorous stirring until the turn of the indicator's color. The addition of the acidic solution was repeated until the stabilization of the indicator in the acid band. The reaction was maintained under stirring overnight, when the TLC analysis indicated the total consumption of the starting material. Then, the reaction mixture was partitioned between ethyl ether (10 mL) and brine. The organic extracts were joined, dried with anhydrous sodium sulfate, and evaporated under reduced pressure to furnish a solid that was subsequently recrystallized in ethanol to produce the desired reduced compound in 52% yield, mp 130–131 °C. ^{1}H NMR (200 MHz): δ 9.97 (d, 1H, CO=N*H*), 6.05 (s, 2H, O–CH_2–O), 5.42 (q, 1H, NH–N*H*–CH_2), 4.18 (d, 2H, NH–CH_2–Ar).

5.2. Pharmacology

The Animal Care and Use Committee at the Universidade Federal do Rio de Janeiro approved the protocol described as follows. Thoracic aorta was dissected from male Wistar rats (240–280 g) and prepared for isometric tension recording. Aorta was cut in 2–3 mm rings and placed in a vertical chamber filled with Tyrode's solution composed of (in mM): NaCl, 120; KCl, 5.9; $MgCl_2$, 1.2; NaH_2PO_4, 0.9; $NaHCO_3$, 24; $CaCl_2$, 2.5; glucose, 11 (pH 7.4) and oxygenated with carbogen gas (95% O_2/5% CO_2) at 37 ± 0.5 °C. Each aorta ring was mounted between two hooks in which one was attached to a force transducer (Grass, mod. FT-03). The transducer signal was conditioned by a Cyberamp (Axon Instruments, Inc.) and then displayed and stored on a computer for future analysis using Axoscope software (Axon Instruments, Inc.). Preparations were stabilized under 1 g resting tension during 2 h and then the contractile response to phenylephrine (10 μM) was measured before and after exposure to increasing concentrations of derivatives. A phenylephrine-induced contracture was followed by exposure to acetylcholine (10 μM) to test the integrity of endothelium. Endothelium was considered intact if acetylcholine-induced relaxation of precontracted aorta was greater than 80%. Removal of functional endothelium was confirmed by the lack of relaxation (<10%) in the presence of acetylcholine. The derivatives were dissolved in dimethyl sulfoxide (DMSO) as stock solutions of 50 mg/mL. Control experiments performed in the presence of DMSO alone, at the same concentrations as those used with the derivatives tested, demonstrated that the solvent did not affect the contractile response of isolated aorta. Phenylephrine and acetylcholine were obtained from Sigma Chemical Co. (St. Louis, MO). All data were expressed as mean of percentage of maximal tension ± SEM. Differences between different concentrations were considered statistically significant when $P < 0.05$ using paired Student's *t*-test. For comparison between two groups with a non-normal distribution, the Mann–Whitney test was used.

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