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Original article

Studies towards the identification of putative bioactive conformation of potent vasodilator arylidene *N*-acylhydrazone derivatives

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ABSTRACT

In this report we disclose the synthesis, vasodilatory activity, and identification of bioactive conformation of new *N*-acylhydrazone and *N*-methyl-*N*-acylhydrazone derivatives, structurally designed by bioisosteric replacements of previously described cardioactive compounds LASSBio-294 and its *N*-methyl derivative LASSBio-785. Some of these novel derivatives presented improved vasorelaxant properties, being new cardiovascular drug candidates.

1. Introduction

Hypertension is the most common cardiovascular disease, and the one which represents the major risk factor for coronary artery disease, heart failure, stroke and renal failure [1]. The reduction in blood pressure achieved with most of the currently used antihypertensive agents, such as angiotensin-converting enzyme inhibitors, calcium antagonists, β-blockers and diuretics, is direct or indirectly related to the relaxation of the vascular smooth muscle [2]. Several direct vasodilators have been synthesized but none of them has presented a specific action while being free of side effects, reinforcing the importance of identifying new clinically safe useful vasodilator agents [3].

The strategy of molecular simplification on pyridazinone phosphodiesterase (PDE) inhibitors (**1**) [4], represented by a simple bond rupture in *a* and the elimination of the stereogenic center in *b*, enabled us to recognize the structural similarity between *N*-acylhydrazone (NAH) derivatives (**2**) and these cardioactive compounds [5]. The inclusion of the 1,3-benzodioxolyl ring, coming from Brazilian natural safrole (**3**) [6] attached to the acyl group of NAH moiety [7,8] and replacing the phenyl ring attached to the imine moiety by the bioisosteric 2-thienyl ring [9] resulted in the design of 3,4-methylenedioxybenzoyl-2-thienyl-hydrazone, named LASSBio-294 (**4**) (Chart 1) [5].

LASSBio-294 (**4**) was described as a potent positive cardioinotropic agent due to an increase in the Ca^{2+} accumulation in the sarcoplasmic reticulum (SR) [10]. In addition, LASSBio-294 (**4**) also promoted vasodilation in aortic rings, mediated by the guanylate cyclase/cyclic guanylate monophosphate pathway [11].

Considering that the *N*-acylhydrazone subunit present in LASSBio-294 (**4**) has an isosteric relationship with the pyridazinone ring shared by phosphodiesterase (PDE) inhibitors [12], the bioprofiles of several synthetic analogues planned by structural optimization of the lead compound LASSBio-294 (**4**) were investigated in the vascular smooth muscle [13]. Among the derivatives tested on the contractile response of the vascular smooth muscle of Wistar rats, we observed that changes in electronic density of the thienyl subunit did not increase the vasodilatory effects [13]. However, the

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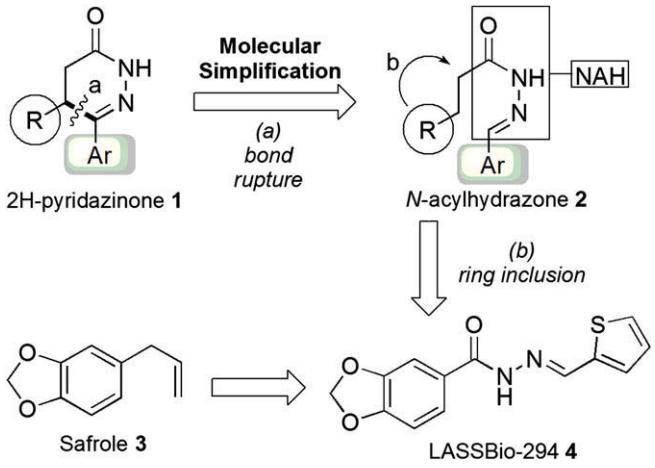


Chart 1. Structural planning of LASSBio-294.

introduction of small alkyl groups linked to the amide nitrogen of the NAH moiety of LASSBio-294 (**4**), especially the methyl group, led to the discovery of derivative LASSBio-785 (**5**), which presented a significant improvement in vasodilator properties (IC_{50} was 10.2 ± 0.5 μM), being seven times more potent than LASSBio-294 (**4**) in the same bioassay [13].

Herein we explore the synthesis of new arylidene *N*-acylhydrazones (**6a–c**) and their corresponding *N*-methyl derivatives (**9a–c**) planned by classical bioisosteric replacement of the thienyl ring from LASSBio-294 (**4**) and LASSBio-785 (**5**) for the phenyl, furanyl and pyrrolyl rings, aiming at determining the structure-associated events of the methyl group to the vasodilator profile of these NAH compounds.

2. Results and discussion

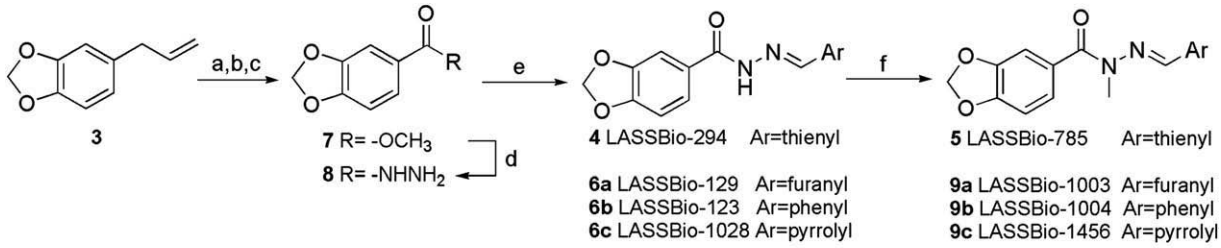
The synthesis of the new *N*-acylhydrazone derivatives (**6a–c**) was carried out as outlined in Scheme 1. The starting material was 3,4-methylenedioxybenzoylhydrazine (**8**) that can be obtained from natural safrole (**3**) in 57% overall yield as described previously [7]. The acid-catalyzed condensation of the hydrazide (**8**) with the corresponding aromatic or heteroaromatic aldehydes resulted in LASSBio-294 (**4**) and its desired isosteric *N*-acylhydrazone derivatives LASSBio-129 (**6a**), LASSBio-123 (**6b**) and LASSBio-1028 (**6c**) in very good yields (Scheme 1). Next, *N*-methyl-NAH series was synthesized using the corresponding *N*-acylhydrazones as precursors after treatment with potassium carbonate in acetone followed by the addition of methyl iodide at 50 °C. Through this procedure we were able to obtain, regioselectively, the *N*-methylated derivatives LASSBio-785 (**5**), LASSBio-1003 (**9a**), LASSBio-1004 (**9b**) and LASSBio-1456 (**9c**) in yields ranging from 88% to 95% after recrystallization.

The careful analysis of ^{1}H NMR spectra of the new acylhydrazone derivatives demonstrated the selective *N*-alkylation by the presence of a single *N*-methyl hydrogen signal ranging from δ 3.4 to 3.6 ppm and the presence of only one imino hydrogen signal ranging from δ 7.7 to 7.9 ppm (*N*-methyl-NAH derivatives) and δ 8.3 to 8.6 ppm (NAH derivatives), attributed to the (*E*)-diastereomer on the basis of several previous reports from our group describing the configuration of bioactive NAH derivatives [7,8,13–17], as well as an only amide hydrogen signal for the NAH derivatives ranging from δ 11.6 to 11.7 ppm.

Vasodilatory activity of these new derivatives was investigated in aortic rings pre-contracted with 10 μM phenylephrine [13]. Fig. 1 shows concentration–response curves for NAH derivatives in aorta with intact endothelium. As it has been shown in LASSBio-294 (**4**) and LASSBio-785 (**5**) [13], the *N*-alkylation of *N*-acylhydrazone compounds, generating the corresponding *N*-methyl-NAH derivatives, led to intense and concentration-dependent aorta relaxation (Fig. 1B). At 20 μM, LASSBio-1004 (**9b**) completely relaxed the phenylephrine-induced contraction, while its non-alkylated analogue (LASSBio-123) (**6b**) produced a relaxation of 70.7 ± 2.7% only at 500 μM. Among the *N*-methyl-NAH derivatives tested, LASSBio-1004 (**9b**) and LASSBio-1456 (**9c**) were more potent than LASSBio-1003 (**9a**) in reducing the phenylephrine-induced contracture with IC_{50} of 3.5 ± 1.1, 3.1 ± 1.1 and 40.8 ± 16.2 μM, respectively (Table 1). Our results indicated that bioisosteric replacements of the aromatic rings bridged to the imine group (*i.e.*, thienyl, pyrrolyl and phenyl) did not drastically change the vasodilator activity, indicating that the interaction of this bioisosteres with a putative receptor would occur mainly through hydrophobic interactions. On the other hand, the furanyl bioisoster (**9a**) was the only *N*-methyl-NAH derivative to present some reduction in activity, may be due to the remarkable electronegative properties of its oxygen atom.

The compounds were also evaluated in aortic rings without a functional endothelium. Removal of the endothelium did not reduce the maximum relaxation and only slightly reduced the vasodilatory potency promoted by *N*-methyl-NAH derivatives (**9a–c**), but abolished the vasodilatory effect of NAH derivative LASSBio-123 (**6b**) until the concentration of 500 μM was reached, as has been seen for LASSBio-294 (**4**) (Table 1), indicating a possible distinct mechanism of action between these two NAH series or an additional vasodilator mechanism for the *N*-methyl-NAH related to the NAH series.

Despite the original structural planning based on the molecular simplification of PDE4 inhibitors, the compounds did not present any significant *in vitro* activity in this enzyme, as well as in the PDE5



[a] Reagents and conditions: (a) KOH aq. 3 N, *n*-BuOH, room temperature, 3 h, 98%; (b) (i) O_3 /O_2, AcOH, 0 °C, 1 h; (ii) Zn^o, AcOH, 93% (75%, 3 steps); (c) I_2, KOH, MeOH, 0 °C, 1.5 h, 90%; (d) $NH_2NH_2.H_2O$, 80%, EtOH, reflux, 3.5 h, 84%; (e) aromatic aldehyde, EtOH, HCl (cat.), room temperature, 30 min, 88-92%; (f) K_2CO_3, acetone, 50°C, 86-95%.

Scheme 1. Synthetic route exploited in the preparation of the target *N*-acylhydrazones (**8**) and their corresponding *N*-alkylated analogues (**9**).

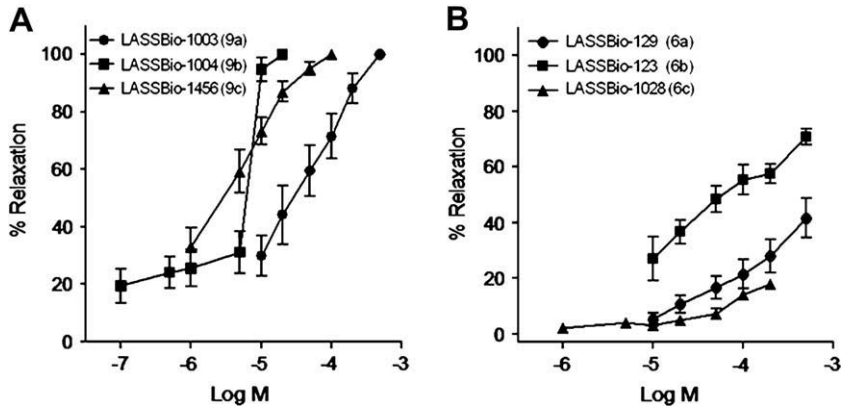


Fig. 1. Effects of NAH derivatives (**6a–c** and **9a–c**) on phenylephrine (Phe)-induced contracture in rat aortic rings. (A) Concentration–response curves for *N*-methyl-NAH derivatives (**6a–c** and **9a–c**) in aorta with intact endothelium. (B) Concentration–response curves for NAH derivatives (**6a–c** and **9a–c**) in aorta with intact endothelium. Data represent mean ± SEM of 8 experiments.

and PDE2A subtypes, showing that this series has a different mechanism of vasodilatation [18].

To best understand the influence of the methyl group for the bioprofile of these NAH derivatives, we performed a structural study by using X-ray diffraction and molecular modeling as tools to investigate possible conformation-associated events.

The X-ray analysis of LASSBio-294 (**4**), LASSBio-785 (**5**), LASSBio-123 (**6b**) and LASSBio-1004 (**9b**) monocrystals obtained from an ethanol saturated solution showed the exclusive formation of (*E*)-diastereomer and the regioselective *N*-methylation of the NAH derivatives as previously attributed in the ^{1}H NMR spectra (Fig. 2).

The most stable structural conformers of the NAH and *N*-methyl-NAH series in the solid state differ between themselves [19]. The first one presented a preferential flat conformation (**I**) (amide hydrogen antiperiplanar to the carbonyl oxygen) while the second one presented a tendency to turn the amide bond by 180° (**II**) (amide hydrogen synperiplanar to the carbonyl oxygen) forming a folded structure (Fig. 2) in a similar manner as described in some aromatic methyl amides [20,21].

The synperiplanar conformation (**II**) adopted after the amide bond 180° rotation is probably due to a steric hindrance introduced by the methyl group in relation to the hydrogen present in position 2 of the 1,3-benzodioxolyl ring (Fig. 2). The preferential folded conformation (**II**) could be explained by a high energy barrier ($\Delta Hf = 17$ kcal/mol) [20] calculated for LASSBio-1004 (**9b**), required for the interconversion of this form (**II**) into the flat antiperiplanar conformer (**I**) (Fig. 2).

In addition, the assumption of conformational differences when in solution was also supported by changes in UV spectra for acetonitrile, between the NAH and *N*-methyl-NAH series. The most remarkable difference between the UV spectra of derivatives belonging to these two series was the antiperiplanar conformation (**I**, Fig. 2) of non-alkylated derivatives, *e.g.* LASSBio-123 (**6b**), which presented absorption at 306 nm ($\log \varepsilon = 4.00$), whereas the antiperiplanar conformation (**I**, Fig. 2) adopted by the corresponding *N*-methyl-NAH derivative LASSBio-1004 (**9b**) shifted it to a shorter wavelength (291 nm) and its absorbance was diminished ($\log \varepsilon = 3.78$) due to a difference in the electronic transition in aromatic moieties between the series caused by conformational variations. These results are in agreement with previous conformational studies of secondary and tertiary aromatic amides [20,22].

3. Conclusions

In this report, we have showed for the first time that the methyl group, bridged to the amide bond of NAH, plays an important role in the vasodilatory activity of these compounds by inducing conformational changes supported by crystallography, molecular modeling and UV studies. The structural modifications introduced by the methyl group are certainly required to the vasodilator effectiveness and potency increase observed for the *N*-methyl-NAH series, as well as to promote changes in its molecular mechanism of action. This investigation enabled the discovery of two new bioisosteric *N*-methyl-NAH prototypes with potent vasodilator properties, *i.e.*, LASSBio-1004 (**9b**) and LASSBio-1456 (**9c**), which were elected for further pharmacological evaluation on *in vivo* hypertension models in order to confirm their potential as antihypertensive drug candidates, as will be disclosed in a forthcoming paper.

4. Experimental

4.1. Synthesis

4.1.1. General information

Melting points were determined with a Quimis 340 apparatus and are uncorrected. ^{1}H NMR spectra were determined in deuterated chloroform or dimethyl sulfoxide containing ca. 1% tetramethylsilane as an internal standard, using a Bruker DPX-200 at 200 MHz. ^{13}C NMR spectra were determined in the same spectrometer at 50 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

Table 1
Effects of NAH derivatives (**4**), (**5**), (**6a–c**) and (**9a–c**) in phenylephrine-induced contracture of aorta.

Compounds	IC_{50} (μM)	
	With endothelium	Without endothelium
N-Acylhydrazones		
LASSBio-123 (**6b**)	81.0 ± 27.2	ND (500 μM)
LASSBio-129 (**6a**)	ND (500 μM)	ND (500 μM)
LASSBio-294 (**4**)	74.0 [11]	ND (100 μM)
LASSBio-1028 (**6c**)	ND (200 μM)	ND (200 μM)
N-Methyl-*N*-acylhydrazones		
LASSBio-785 (**5**)	10.2 ± 0.5 [13]	18.5 ± 3.6
LASSBio-1003 (**9a**)	40.8 ± 16.2	15.7 ± 4.4
LASSBio-1004 (**9b**)	3.5 ± 1.1	11.1 ± 0.9*
LASSBio-1456 (**9c**)	3.1 ± 1.1	17.8 ± 7.2

ND = not determined, because the maximal concentration tested did not yield 50% of vasorelaxant activity. Numbers in brackets indicate the maximal concentration tested.
*$P < 0.05$ compared to with endothelium.

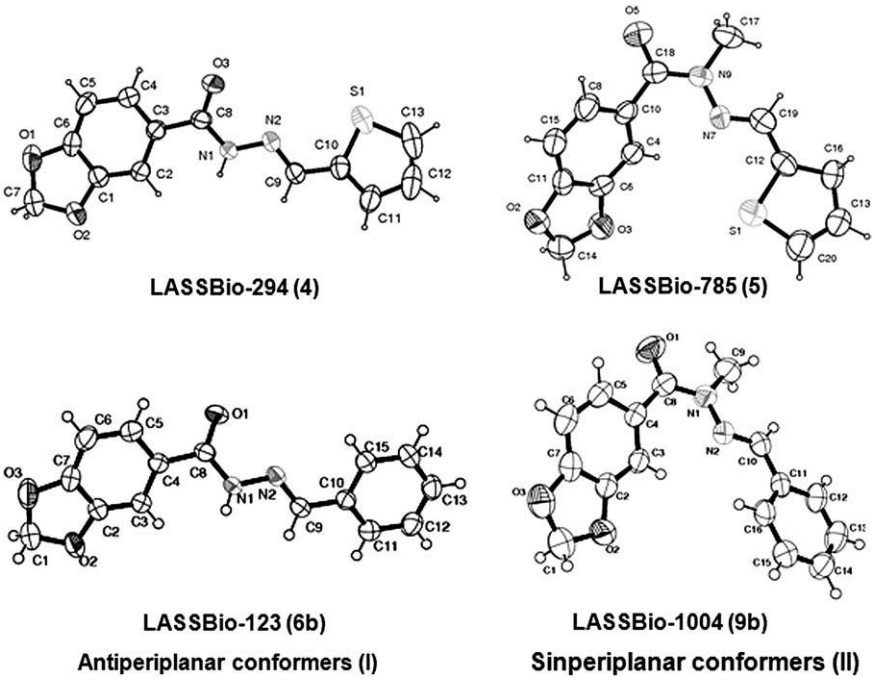


Fig. 2. ORTEP view of representative NAH and *N*-methyl-NAH derivatives LASSBio-294 (**4**), LASSBio-785 (5), LASSBio-123 (6b) and LASSBio-1004 (9b) showing the alkylation-associated conformational changes.

thin layer chromatography, 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. Reagents and solvents were purchased from commercial suppliers and used as received.

*4.1.2. General procedure for the preparation of 3,4-methylenedioxybenzoyl-acylhydrazones LASSBio-123 (**6b**), LASSBio-129 (**6a**), LASSBio-294 (**4**), and LASSBio-1028 (**6c**)*

To a solution of 3,4-methylenedioxybenzoylhydrazine (**8**) (0.150 g, 0.83 mmol), prepared as previously described [7], in absolute EtOH (7 mL) containing two drops of 37% hydrochloric acid, 0.87 mmol of the corresponding aromatic or heteroaromatic aldehyde derivative was added. The mixture was stirred at room temperature for 30 min until extensive precipitation was visualized. Afterwards, 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 producing the desired acylhydrazone derivatives LASSBio-123 (**6b**), LASSBio-129 (**6a**), LASSBio-294 (**4**), and LASSBio-1028 (**6c**), as described below.

*4.1.2.1. (Benzylidene)3,4-methylenedioxybenzoylhydrazine (LASSBio-123) (**6b**).* Derivative LASSBio-123 (**6b**) was obtained as a white solid by condensation of **8** with benzaldehyde in 92% yield, mp 180–181 °C. ^{1}H NMR data are in agreement with those previously published [7]. MS *m*/*z*: 268 (M+, 5%), 165 (38%), 149 (100%), 121 (17%), 103 (5%). Anal. Calcd for $C_{15}H_{12}N_2O_3 \cdot H_2O$: C 62.93, H 4.93, N 9.79; found: C 62.88, H 4.91, N 9.48.

*4.1.2.2. (2-Furanylidene)3,4-methylenedioxybenzoylhydrazine (LASSBio-129) (**6a**).* Derivative LASSBio-129 (**6a**) was obtained as a cream solid by condensation of **8** with 2-furanecarboxyaldehyde in 88% yield, mp 215–216 °C. ^{1}H NMR (200 MHz) DMSO-d_6: δ 11.66 (s, 1H, CONH–), 8.32 (s, 1H, =CH), 7.84 (d, 1H, H_5', $J = 1.3$ Hz), 7.50 (dd, 1H, H_6, $J_{1-2} = 8.2$ Hz and $J_{1-3} = 1.4$ Hz), 7.43 (d, 1H, H_2, $J = 1.4$ Hz), 7.04 (d, 1H, H_5, $J = 8.2$ Hz), 6.91 (d, 1H, H_3', $J = 3.2$ Hz), 6.63 (dd, 1H, H_4', $J_{1-2} = 3.2$ Hz and $J_{1-3} = 1.3$ Hz), 6.12 (s, 2H, O–CH_2–O). MS *m*/*z*: 258 (M+, 12%), 165 (20%), 149 (100%), 121 (22%), 93 (5%). Anal. Calcd for $C_{13}H_{10}N_2O_4$: C 60.47, H 3.90, N 10.85; found: C 60.65, H 3.88, N 10.65.

*4.1.2.3. (2-Thienylidene)3,4-methylenedioxybenzoylhydrazine (LASSBio-294) (**4**).* Derivative LASSBio-294 (**4**) was obtained as a white solid by condensation of **8** with 2-thiophenecarboxyaldehyde in 90% yield, mp 204–205 °C. ^{1}H NMR data are in agreement with those previously published [13]. MS *m*/*z*: 274 (M+, 9%), 165 (32%), 149 (100%), 121 (12%), 109 (6%). Anal. Calcd for $C_{13}H_{10}N_2O_3S \cdot H_2O$: C 53.42, H 4.14, N 9.58; found: C 53.34, H 4.11, N 9.25.

*4.1.2.4. (2-Pyrrolidene)3,4-methylenedioxybenzoylhydrazine (LASSBio-1028) (**6c**).* Derivative LASSBio-1028 (**6c**) was obtained as a cream solid by condensation of **8** with 2-pyrrolecarboxyaldehyde in 88% yield, mp 181–182 °C. ^{1}H NMR (200 MHz) DMSO-d_6: δ 11.57 (s, 1H, CONH–), 11.42 (s, 1H, NH-pyrrol), 8.20 (s, 1H, =CH), 7.52 (d, 1H, H_6, $J = 8.2$ Hz), 7.40 (s, 1H, H_2), 7.02 (d, 1H, H_5, $J = 8.2$ Hz), 6.92 (d, 1H, H_5', $J = 1.3$ Hz), 6.48 (d, 1H, H_3', $J = 3.2$ Hz), 6.18 (dd, 1H, H_4', $J_{1-2} = 3.2$ Hz and $J_{1-3} = 1.3$ Hz), 6.12 (s, 2H, O–CH_2–O). Anal. Calcd for $C_{13}H_{11}N_3O_3 \cdot H_2O$: C 56.76, H 4.76, N 15.27; found: C 56.86, H 4.75, N 14.47.

4.1.3. General procedure for selective N*-methylation of compounds LASSBio-123 (**6b**), LASSBio-129 (**6a**), LASSBio-294 (**4**) and LASSBio-1028 (**6c**)*

The correspondent *N*-acylhydrazone (1.0 mmol) and potassium carbonate (3.0 mmol) were suspended in 5 mL of acetone. The suspension was thoroughly mixed under vigorous stirring for 5 min and then methyl iodide (3.0 mmol) was added. The reaction was heated at 50 °C and maintained under stirring for 24 h. Afterwards, the reaction was evaporated under reduced pressure, the residual solid suspended in 2 mL of ethanol and then poured into cold water. The precipitate formed was filtered out, dried under vacuum and

washed with petroleum ether producing the desired *N*-methyl-*N*-acylhydrazone derivatives LASSBio-785 (**5**), LASSBio-1003 (**9a**), LASSBio-1004 (**9b**), and LASSBio-1456 (**9c**), as described below.

4.1.3.1. N-*Methyl (2-thienylidene)3,4-methylenedioxybenzoylhydrazine (LASSBio-785) (**5**).* Derivative LASSBio-785 (**5**) was obtained as a white solid by *N*-alkylation of LASSBio-294 (**4**) with methyl iodide, in 92% yield, mp 142–143 °C. ^{1}H NMR data are in agreement with those previously published [13]. MS *m*/*z*: 288 (M+, 5%), 179 (15%), 149 (100%), 121 (13%), 109 (4%). Anal. Calcd for $C_{14}H_{12}N_2O_3S$: C 58.32, H 4.20, N 9.72; found: C 58.39, H 4.19, N 9.34.

4.1.3.2. N-*Methyl (2-furanylidene)3,4-methylenedioxybenzoylhydrazine (LASSBio-1003) (**9a**).* Derivative LASSBio-1003 (**9a**) was obtained as a white cream solid by *N*-alkylation of LASSBio-129 (**6a**) with methyl iodide, in 89% yield, mp 104–105 °C. ^{1}H NMR (200 MHz) $CDCl_3$: δ 7.66 (s, 1H, =CH), 7.47 (d, 1H, H_5', $J = 1.8$ Hz), 7.42 (dd, 1H, H_6, $J_{1-2} = 8.2$ Hz and $J_{1-3} = 1.4$ Hz), 7.34 (d, 1H, H_2, $J = 1.4$ Hz), 6.85 (d, 1H, H_5, $J = 8.2$ Hz), 6.62 (d, 1H, H_3', $J = 3.3$ Hz), 6.45 (dd, 1H, H_4', $J_{1-2} = 3.3$ Hz and $J_{1-3} = 1.8$ Hz), 6.02 (s, 2H, O–CH_2–O), 3.50 (s, 1H, N–CH_3). MS *m*/*z*: 272 (M+, 9%), 179 (14%), 149 (100%), 121 (15%), 93 (5%).

4.1.3.3. N-*Methyl (benzylidene)3,4-methylenedioxybenzoylhydrazine (LASSBio-1004) (**9b**).* Derivative LASSBio-1004 (**9b**) was obtained as a white solid by *N*-alkylation of LASSBio-123 (**6b**) with methyl iodide, in 95% yield, mp 109–110 °C. ^{1}H NMR (200 MHz) $CDCl_3$: δ 7.75 (s, 1H, =CH), 7.55 (d, H_2', $J = 4.0$ Hz), 7.35–7.27 (m, 5H, H_2, H_6, H_3', H_4'), 6.86 (d, H_5, $J = 8.2$ Hz), 6.03 (s, 2H, O–CH_2–O), 3.54 (N–CH_3). MS *m*/*z*: 282 (M+, 6%), 179 (17%), 149 (100%), 121 (15%), 103 (5%). Anal. Calcd for $C_{16}H_{14}N_2O_3$: C 68.07, H 5.00, N 9.92; found: C 67.92, H 4.99, N 9.54.

4.1.3.4. N-*Methyl (2-pyrrolidene) 3,4-methylenedioxybenzoylhydrazine (LASSBio-1456) (**9c**).* Derivative LASSBio-1456 (**9c**) was obtained as a cream solid by *N*-alkylation of LASSBio-1028 (**6c**) with methyl iodide, in 86% yield, mp 106–107 °C. ^{1}H NMR (200 MHz) $CDCl_3$: δ 10.82 (s, 1H, NH-pyrrol), 7.87 (s, 1H, =CH), 7.23 (dd, 1H, H_6, $J_{1-2} = 8.2$ Hz and $J_{1-3} = 1.4$ Hz), 7.17 (d, 1H, H_2, $J = 1.4$ Hz), 6.94 (d, 1H, H_5, $J = 8.2$ Hz), 6.85 (d, 1H, H_5', $J = 1.5$ Hz), 6.40 (d, 1H, H_3', $J = 3.2$ Hz), 6.11 (dd, 1H, H_4', $J_{1-2} = 3.2$ Hz and $J_{1-3} = 1.5$ Hz), 6.07 (s, 2H, O–CH_2–O), 3.41 (s, 1H, N–CH_3). MS *m*/*z*: 271 (M+, 8%), 179 (15%), 149 (100%), 121 (14%), 92 (9%). Anal. Calcd for $C_{14}H_{13}N_3O_2$: C 61.99, H 4.83, N 15.49; found: C 61.51, H 4.82, N 14.97.

4.2. 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 rings of 2–3 mm 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 and 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 in a computer for future analysis using Axoscope software (Axon Instruments, Inc.). Preparations were stabilized under 1 g resting tension for 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 the endothelium. The endothelium was considered intact if acetylcholine-induced relaxation of pre-contracted 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 test. For comparison between two groups with a non-normal distribution, the Mann–Whitney test was used.

4.3. X-ray crystallography

Data pertaining to the X-ray crystallographic determination of **4**, **5**, **6b** and **9b** have been deposited with the Cambridge Crystallographic Data Centre 60 H.N. as supplementary publication no.: LASSBio-123 (**6a**) CCDC 707595; LASSBio-294 (**4**) CCDC 707596; LASSBio-785 (**5**) CCDC 707597; and LASSBio-1004 (**9b**) CCDC 707598. This information may be freely obtained by applying to CCDC, 12 Union Road, Cambridge CB2 1EZ, UK (fax: +44 (0) 1223 336033 or e-mailing to deposit@ccdc.cam.ac.uk).

4.4. Molecular modeling

The sketching, geometry optimization and conformational search of compounds were performed in BioMedCAche 6.0 software [20]. The local energy minimization was obtained with semiempirical AM1 method followed by application of the same method for a global minimization using dihedral angle search. The energy barrier introduced by the methyl group was calculated using AM1 method with a dihedral angle search in 24 steps of 15° on the amide bond.

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Appendix. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ejmech.2009.04.044.

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