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Original article

Synthesis and anti-platelet activity of novel arylsulfonate–acylhydrazone derivatives, designed as antithrombotic candidates

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Abstract

In this work, we describe a new class of promising anti-platelet drug candidates with significant antithrombotic activity in vivo. This new series of compounds was structurally planned by modification of known thrombin inhibitors based on the use of acylhydrazone subunit, as a non-peptide scaffold, and variations at P1 moiety. Three different families of arylsulfonate–acylhydrazone derivatives were designed. The bioassays indicated the first class of derivatives represented by **4f** (LASSBio-693) and **4j** (LASSBio-743), which were active in inhibiting the platelet aggregation induced by thrombin. The second class represented by compounds **4e** (LASSBio-774) and **4h** (LASSBio-480) that selectively inhibit the platelet aggregation involving TXA_2 formation. Finally, the third class of derivatives was identified acting as a novel symbiotic agent able to inhibit the platelet aggregation induced by collagen or AA and by thrombin, represented by compounds **4b** (LASSBio-694) and **4g** (LASSBio-770).

Keywords: Anti-platelet activity; *N*-Acylhydrazone (NAH); Thrombin

1. Introduction

Thromboembolic disorders are the major cause of morbidity and mortality in Western societies. Clinical regulation of thrombosis involves the anti-platelet therapy (e.g. acetylsalicylic acid), thrombolytic agents (e.g. streptokinase), and drugs that affect the activity and generation of thrombin (e.g. heparin, argatroban) [1–3].

Thrombin is a multifunctional serine protease that plays a central role in thrombosis and hemostasis, not only by enzymatic activation of coagulation factors, but also by direct stimulation of several cell types such as platelets [4,5].

Platelets are also involved in the thrombotic pathologies. They play a major role in vascular occlusive diseases such as angina, myocardial infarction, and stroke, as a consequence of their inappropriate and sustained activation, being thus considered also important targets for antithrombotic drugs [6]. Inhibitors of thrombin have long been recognized as potential therapeutic agents for the treatment of a variety of thrombotic disorders [7]. In fact, intravenous (e.g. argatroban) and oral (e.g. ximelagatran) chiral thrombin inhibitors have shown promising results in human clinical trials [7,8]. It was often observed that thrombin inhibitors with strongly basic P1 moieties have low selectivity against other serine proteases and poor oral bioavailability. Therefore, derivatives bearing P1 elements with modulated basicity (between pKa 6.5 and 9.0) may be considered more promising lead-compounds [9].

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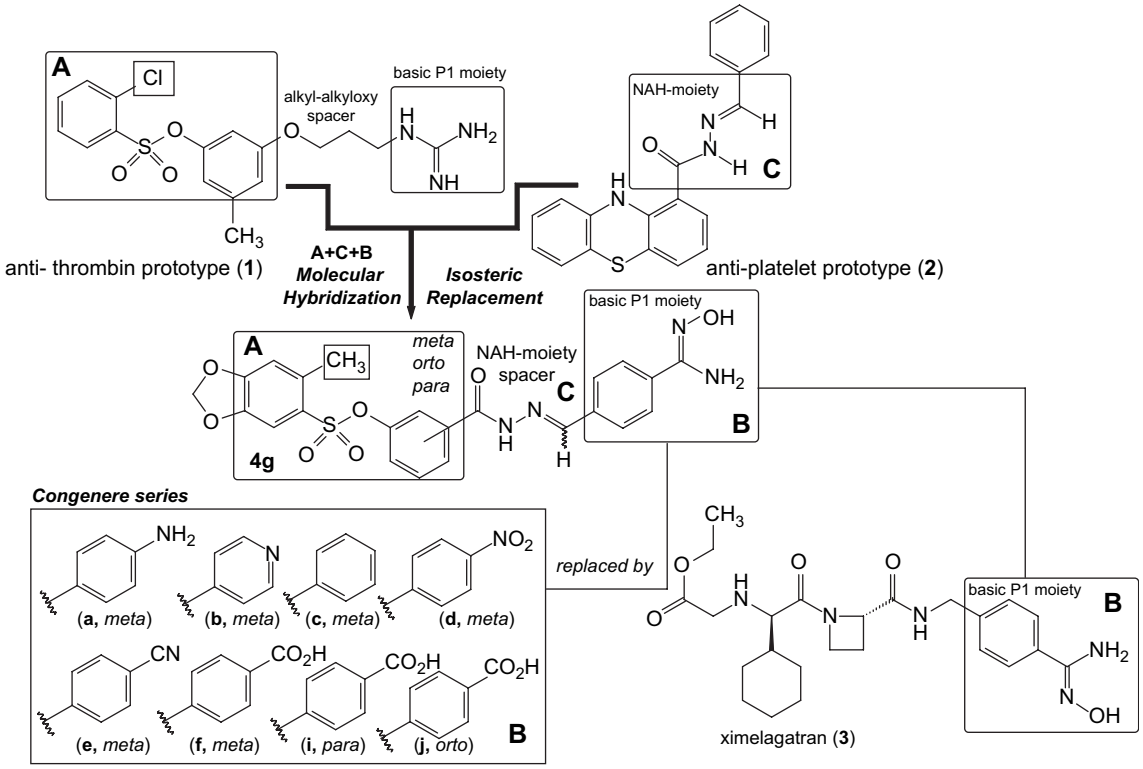
In this paper, we report the design, synthesis and anti-platelet activity of novel arylsulfonate–acylhydrazone derivatives (**4a–j**). These compounds were planned by applying molecular hybridization strategy on classical thrombin inhibitors (**1**) [11] and on anti-platelet *N*-acylhydrazone (NAH) compound (**2**) [12], elected as prototypes (Scheme 1). The main structural features of the new arylsulfonate–acylhydrazone derivatives (**4a–j**) were based on classic isosteric replacement [20] of chlorine atom, of the structure of anti-thrombin prototype (**1**), by methyl group and the introduction of the NAH moiety instead of the alkyl–alkyloxy subunit present in **1**. Otherwise, considering the aza–vinylogue relationship of NAH group with the amide present in the structures of ximelagatran (**3**), it seems reasonable to introduce the NAH moiety as a spacer between the subunits arylsulfonate (**A**) and *N*-hydroxy benzamidine (**B**) (Scheme 1). Finally, variations at terminal basic *N*-hydroxy-benzamidine subunit **B** by groups like aniline and pyridine were done in order to obtain compounds bearing P1 moiety with modulated basicity. Otherwise, the chemical nature of the subunit **B** can also be modified aiming to investigate the contribution of neutral and acid groups in the activity of this new series of compounds, designed as antithrombotic candidates.

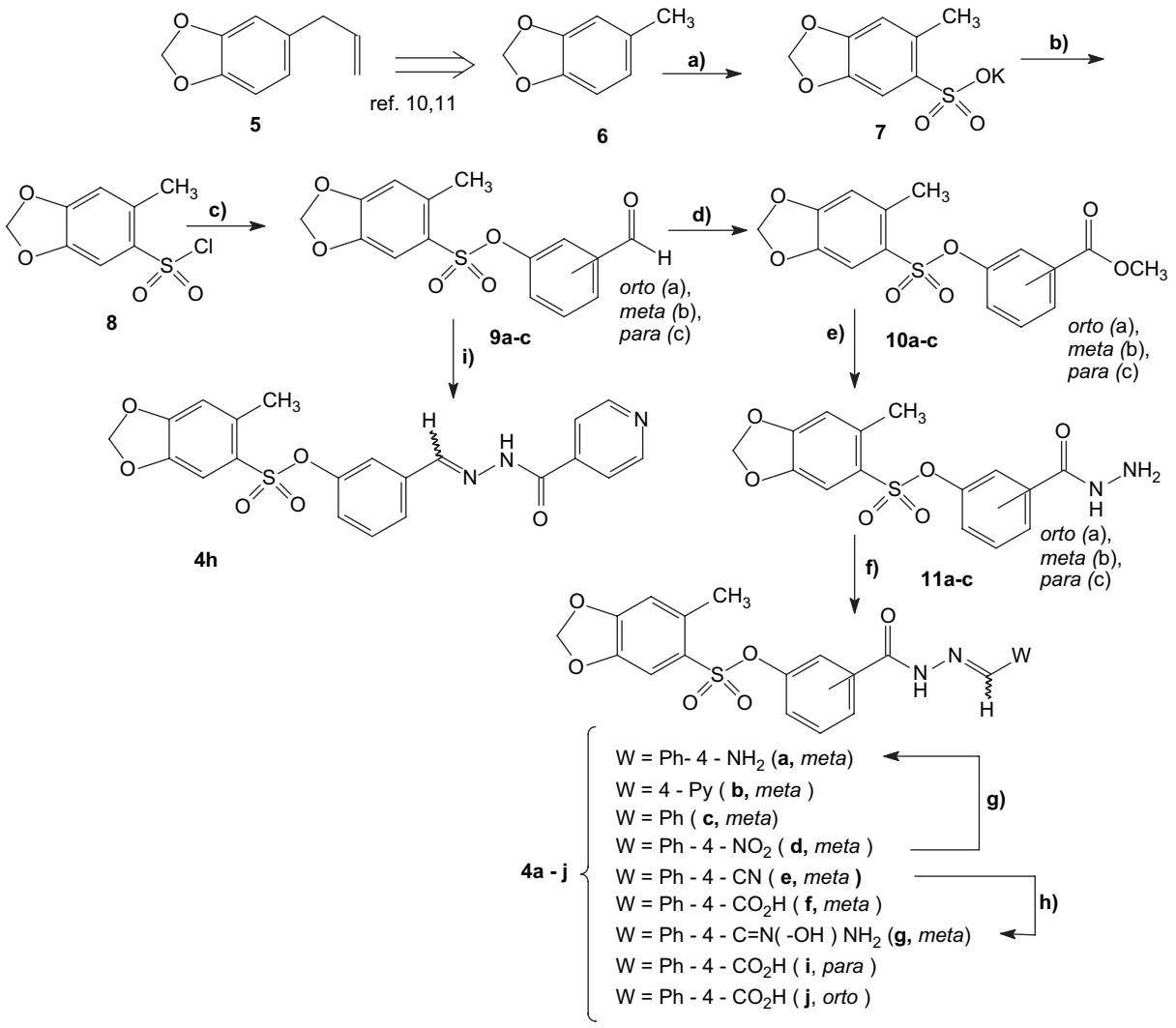
2. Chemistry and biological activity testing

The target derivatives (**4a–j**) were synthesized as depicted in Scheme 2. In the synthetic approach compound 2-methyl-4,5-methylenedioxy-phenyl-sulfonyl chloride (**8**) was identified as the key precursor. This compound can be obtained by regioselective aromatic electrophilic substitution at the C-6 position of 3,4-methylenedioxytoluene (**6**), which was obtained in ca. 50% overall yield from natural safrole (**5**), an abundant natural product [12,13]. As previously described [10], simple distillation of sassafras oil generates **5** in 85% yield. Basic catalyzed isomerization of the double bond followed by oxidative cleavage and Wolff–Kishner reduction gave **6** in appropriated yield. The arylsulfonyl chloride derivative (**8**) was next prepared in two steps from **6**, applying an efficient and mild sulfonation of 1,3-benzodioxole derivative **6** followed by the treatment of the potassium salt (**7**) with thionyl chloride, catalyzed by the Vilsmeyer–Haack complex, to afford derivative **8** in 74% yield [13].

With an attractive method for access to the key intermediate (**8**), we next performed the condensation of this compound with phenols (e.g. 3-hydroxybenzaldehyde, 4-hydroxybenzaldehyde and 2-hydroxybenzaldehyde) in chloroform at room temperature, in the presence of triethylamine, to afford the corresponding arylsulfonate–aldehyde derivatives **9a–c** in 81–98% yield. The synthesis of NAH-derivatives (**4b–f**, **4i**, **4j**) was carried out by exploring successive classical functional groups interconversions on compounds **9a–c**. Thus, the treatment of **9a–c** with sodium cyanide and manganese oxide in methanol at room temperature [14] furnished the ester intermediates (**10a–c**) in 60–97% yield. The hydrazinolysis of the methyl esters (**10a–c**) furnished the corresponding hydrazide derivatives (**11a–c**) in 75–81% yield. Condensation of **11a–c** with the appropriate aromatic aldehydes yielded the new series of sulfonate–acylhydrazone derivatives (**4b–f**, **4i**, **4j**) in excellent yield. Analysis of the ^{1}H NMR spectra of the new



Scheme 1.



Scheme 2. Conditions: (a) (1) H_2SO_4, Ac_2O, AcOEt, 0 °C, r.t., 2 h, (2) AcOK, EtOH, r.t., 30 min, 91%; (b) SO_2Cl, DMF cat, reflux, 4 h, 74%; (c) 3-hydroxybenzaldehyde (or 2-hydroxybenzaldehyde or 4-hydroxybenzaldehyde), Et_3N, CH_2Cl_2, r.t., 2 h, 81–98%; (d) MnO_2, NaCN, MeOH, r.t., 24 h, 60–97%; (e) $N_2H_4 \cdot H_2O$, EtOH, r.t., 2 h, 75–81%; (f) ArCHO, EtOH, HCl cat, reflux, 1 h, 84–98%; (g) Fe, NH_4Cl, EtOH/H_2O (2:1), reflux, 30 min, 75%; (h) $NH_2OH \cdot HCl$, Na_2CO_3, EtOH, H_2O, reflux, 8 h, 69%; (i) isoniazide, EtOH, HCl cat, r.t., 30 min,78%.

derivatives **4b–f** and **4i**, **4j** showed the presence of a single signal referring to ylidenic hydrogen, attributed to the *E*-diastereomer in agreement with the previous report [15]. Finally, compounds **4a** and **4g** were prepared by interconvertion of nitro and nitrile groups, respectively, as follow. Compound **4a** was prepared by reduction of derivative **4d** in the presence of iron powder and ammonium chloride in a mixture of ethanol and water [16], while compound **4g** was synthesized by the treatment with hydroxylamine in ethanol at reflux in 69% yield [17]. Finally, compound **4h** was prepared in good yield by condensation of hydrazide **9a** with isoniazide, using hydrochloric acid as catalyst. The analysis of the ^{1}H NMR spectra of the hydrazone derivative **4h** showed the presence of two ylidenic hydrogen signals at δ 7.9 and 8.5 attributed to the *Z*- and *E*-diastereomers (1:4), respectively.

The in vitro anti-platelet activity of these novel arylsulfonate–acylhydrazone derivatives (**4a–j**, 150 μM) was evaluated by their ability to inhibit platelet aggregation of rabbit platelet-rich plasma (PRP) [18] induced by thrombin (2 nM), ADP (5 μM), collagen suspension (5 μg.mL^{-1}) and arachidonic acid (AA) (100 μM), using acetylsalicylic acid (AAS, 150 μM) as a positive control.

3. Results and discussion

The primary screening data for all compounds (150 μM) are shown in Table 1. The results, displayed in Table 1, demonstrate that compounds **4b** (LASSBio-694) and **4g** (LASSBio-770) having a basic P1 subunit (pyridine and *N*-hydroxybenzamidine, respectively) were able to inhibit the platelet aggregation induced by thrombin in 72.5% and 81%, respectively, while compound **4a** (LASSBio-751), having aminophenyl P1 subunit, was inactive. Nevertheless, the anti-platelet activity found for these compounds, when the platelets were induced either by AA or collagen, indicates that **4b** and **4g** could have dual mechanism of action. In fact, its known

Table 1
Effect of sulfonate-acylhydrazone derivatives (**4a–j**) on in vitro platelet aggregation of citrated rabbit platelet-rich plasma induced by thrombin, arachidonic acid, ADP and collagen

Compound	Concentration (μM)	*n*	Thrombin (2 nM)		*n*	Arachidonic acid (100 μM)		*n*	ADP (5 μM)		*n*	Collagen (5 μg/mL)		*n*
			Aggregation (%)	Inhibition (%)		Aggregation (%)	Inhibition (%)		Aggregation (%)	Inhibition (%)		Aggregation (%)	Inhibition (%)	
Control		4	59.5 ± 1.3		5	76.0 ± 1.4		4	81.2 ± 4.9		4	78.7 ± 3.2		4
AAS	150	3	10.3 ± 6.4	82.7*	3	21.6 ± 4.5	71.6*	3	74.8 ± 0.2	92.1*	3	17.5 ± 12.3	77.7*	3
4a (LASSBi0-751)	150	3	58.2 ± 2.2	2.2	3	15.2 ± 2.0	80*	3	100 ± 0.0	0	3	49.2 ± 3.2	37.5*	3
4b (LASSBio-694)	150	3	16.4 ± 5.2	72.5*	4	13.7 ± 3.5	82*	3	100 ± 0.0	0	3	59.0 ± 0.2	25	3
4c (LASSBio-744)	150	3	100 ± 0.0	0	3	2.2 ± 2.8	97*	3	100 ± 0.0	0	3	45.6 ± 6.3	42*	3
4d (LASSBio-750)	150	3	57.7 ± 2.2	3	3	14.1 ± 3.1	81.5*	3	100 ± 0.0	0	3	69.6 ± 3.5	11.5	3
4e (LASSBio-774)	150	3	58.3 ± 2.0	2	3	0.0 ± 0.0	100*	3	54.9 ± 0.3	32	3	0.0 ± 0.0	100*	3
4f (LASSBio-693)	150	3	2.9 ± 3.0	95*	4	100 ± 0.0	0	3	100 ± 0.0	0	3	100 ± 0.0	0	3
4g (LASSBio-770)	150	3	11.3 ± 0.58	81*	3	0.0 ± 0.0	100*	3	58.7 ± 7.9	36	3	0.0 ± 0.0	100*	3
4h (LASSBio-480)	150	3	58.7 ± 1.3	1.3	3	4.0 ± 1.6	94.8*	3	63.3 ± 6.9	22	3	0.0 ± 0.0	100*	3
4i (LASSBio-752)	150	3	0.0 ± 0.0	100*	3	6.9 ± 4.1	90.8*	3	100 ± 0.0	0	3	59.0 ± 4.0	25	3
4j (LASSBio-743)	150	3	11.9 ± 3.5	80*	3	100 ± 0.0	0	3	100 ± 0.0	0	3	100 ± 0.0	0	3

n = Number of independent experiments carried out in triplicate; *$p < 0.05$ compared to appropriate control (Student's *t* test).

that in rabbit PRP, the collagen and AA induced aggregation is mediated by thromboxane (TXA_2) formation [19]. Intriguing, the evaluation of the retroisoster [20] of compound **4b**, derivative **4h** (LASSBio-480), revealed loss of activity upon thrombin induced platelet aggregation and potentiated the anti-platelet activity mediated by AA or collagen. As anticipated, none of the derivatives having a neutral P1 subunit was active in inhibiting the aggregation induced by thrombin. However, compounds **4c** (LASSBio-744), **4d** (LASSBio-750) and **4e** (LASSBio-774) effectively inhibited the platelet aggregation induced by AA and collagen, indicating a distinct profile when compared to **4b** and **4g** (Table 1). Curiously, derivative **4f** (LASSBio-693), having an acidic P1 subunit, showed a great selectivity and activity on inhibition of platelet aggregation induced by thrombin (95% inhibition). With these results in hands, the regioisomers of compound **4f** (*meta*-isomer), i.e., derivatives **4i** (*para*-isomer) and **4j** (*ortho*-isomer), were synthesized and evaluated with the same bioassay (Table 1). As showed in Table 1, the replacement of *N*-acylhydrazone moiety at the position 3 (*meta*, **4f**) either to the position 4 (*para*, **4i**) or the position 2 (*ortho*, **4j**) results in activity on thrombin level, although the compound **4j** loses the selectivity. These results indicated that geometric modifications, exploring the space relationship between the NAH and sulfonate subunits, can address the activity on different physiological agonists of platelet aggregation process. Otherwise, electronical parameters also seem to be important and only compounds bearing basic or acid groups at the P1 subunit were able to prevent platelet aggregation induced by thrombin.

Molecular modeling studies were carried out using SYBYL molecular mechanics in order to establish the conformer distribution in compounds **4f** (LASSBio-693), **4i** (LASSBio-752) and **4j** (LASSBio-743). Then, the global minimum conformer of **4f**, **4i** and **4j** were selected to perform the equilibrium geometry using the semiempirical AM1 Hamiltonian (Table 2) [21].

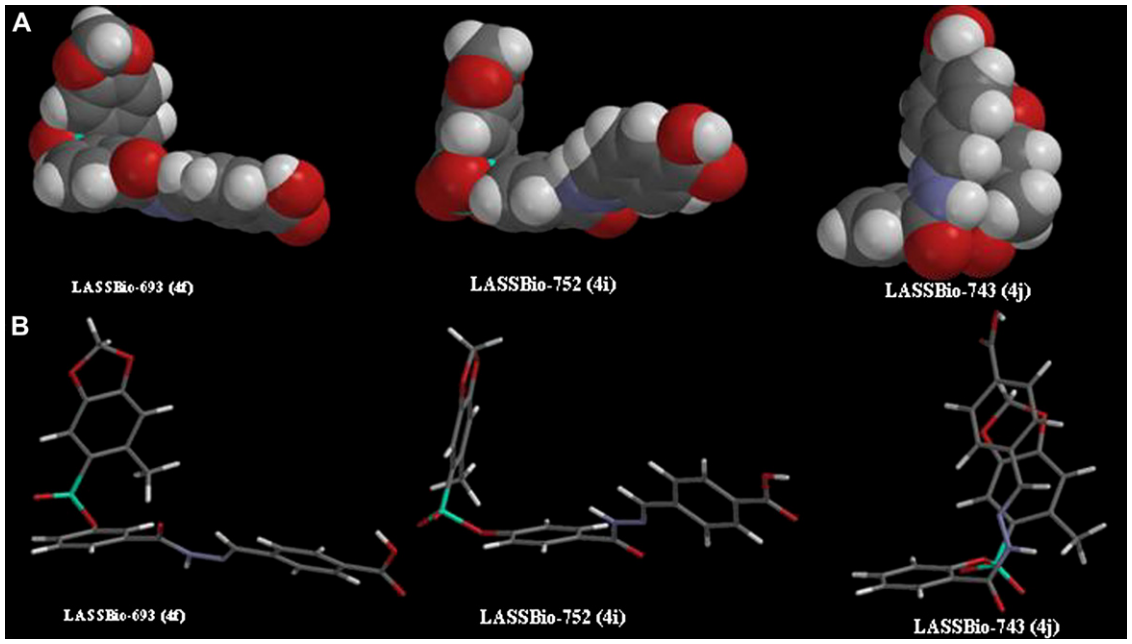
The results from conformational analysis of derivatives **4f**, **4i** and **4j** provided a rational for the differences found in the pharmacological profile of *ortho*-isomer **4j**, when compared to *meta*- **4f** and *para*-isomers **4i** (Table 2). As shown in Table 2, these regioisomers also differ in their molecular shape, area and volume, having distinct polarity. Compounds **4f** and **4i** are more similar in this series.

More attractive compounds **4b**, **4f**, **4i** and **4j** were selected to evaluate their antithrombotic effects in a pulmonary thromboembolism model induced by thrombin in mouse [23]. As shown in Table 3, all compounds were able to increase the survival rate when given orally at a dose of 100 μmol/kg.

4. Conclusions

In summary, we demonstrated that the novel series of arylsulfonate–acylhydrazone derivatives (**4a–j**) represent a promising anti-platelet lead-candidate with significant antithrombotic activity in vivo. Structural modifications at P1 subunit exploring neutral, acid or basic groups can address the activity on the platelet, resulting in compounds that are more or less selective on thrombin levels. Otherwise, the results from the bioassays

Table 2
Minimum energy conformer of arylsulfonate–acylhydrazone derivatives (**4f**, **4i** and **4j**). (**A**) Space-filling (CPK) model for compounds **4f**, **4i** and **4j**; **B**) tube model for compounds **4f**, **4i** and **4j**



Compound	ΔH_f (kcal/mol)	Diastereomer	Conformer	Area (A^2)	Volume (A^3)	Dipole (debye)
4f	−169.23	*E*	*S-cis*	494.02	488.74	7.012
4i	−169.55	*E*	*S-trans*	498.20	489.81	9.121
4j	−163.71	*E*	*S-cis*	490.28	488.01	4.648

identify a new class of derivatives, the first class, represented by compounds **4f** and **4j**, which were active in inhibiting the platelet aggregation induced by thrombin, the second class of derivatives, represented by compounds **4e** and **4h**, that inhibit the platelet aggregation involving TXA_2 formation and finally the third class of derivatives acting as a novel symbiotic candidates, able to inhibit simultaneously the platelet aggregation induced by collagen or AA and by thrombin, represented by compounds **4b** and **4g**.

5. Experimental

Melting points were determined with a Quimis 340 apparatus and are uncorrected. ^{1}H NMR spectra, unless otherwise stated, were obtained in deuterated dimethylsulfoxide or deuterated chloroform containing ca. 1% tetramethylsilane as an internal standard using a Bruker AC 200 spectrometer at 200 MHz. Splitting patterns are as follows: s, singlet; d, doublet; dd, double doublet; dt, double triplet; br, broad; m, multiplet. ^{13}C NMR spectra were obtained using the same spectrometer described above at 50 MHz, using deuterated dimethylsulfoxide as internal standard. IR spectra were obtained using Bruker IFS66 spectrophotometer employing potassium bromide plates. Microanalysis data were obtained using a Perkin-Elmer 240 analyser, using a Perkin-Elmer AD-4 balance. The progress of all reactions was monitored by TLC performed on 2.0 × 6.0 cm aluminium sheets precoated with silica gel 60 (HF-254, E. Merck) to a thickness of 0.25 mm. The developed chromatograms were viewed under ultraviolet light at 254 nm. E. Merck silica gel (60–200 mesh) was used for column chromatography.

5.1. Anti-platelet activity

Blood was withdrawn from rabbit ear central artery and mixed with 3.8% trisodium citrate (9:1 v/v). Platelet-rich plasma (PRP) was prepared by centrifugation at 375 × g for 10 min at room temperature. The platelet-poor plasma (PPP) was prepared by centrifugation of the pellet at 1800 × g for 10 min at room temperature. Platelet aggregation was monitored by the turbidimetric method of Born and Cross [18] using a Chrono-Log aggregometer. PRP (300 μL) was incubated

Table 3
Antithrombotic effect of compounds (**4b**, **4f**, **4j** and **4i**) in a pulmonary thromboembolism model induced by thrombin in mouse

Compound	Dose (p.o.) μmol/kg (0.1 mL/20 g)	Survive (%)
Control	–	16.67 ± 3.33
4b (LASSBio-694)	100	33.33 ± 6.67
4f (LASSBio-693)	100	53.33 ± 6.67
4j (LASSBio-743)	100	80.00 ± 11.5
4i (LASSBio-752)	100	80.00 ± 1.00

at 37 °C for 1 min with continuous stirring at 900 rpm. Platelet aggregation was induced by ADP (5 μM), collagen suspension (5 μg.mL^{-1}), thrombin (2 nM) or arachidonic acid (AA, 100 μM). Compound (150 μM) or vehicle DMSO (0.5% v/v) was added to the PRP samples 1 min before addition of the aggregating agent. Acetylsalicylic acid (AAS, 150 μM), a classical PGHS inhibitor, was used as positive control.

5.2. Pulmonary thromboembolism model induced by thrombin in mouse [23]

Arylsulfonate–acylhydrazones derivatives (**4b**, **4f**, **4j** and **4i**) were given orally (100 μmol/kg; 0.1 mL/20 g) as a suspension in 5% arabic gum in saline (vehicle), 60 min before i.v. injection of the thrombogenic stimulus. Thrombotic event was induced by human thrombin (2000 UI/kg animal) and the survival rate was evaluated 15 min after thrombotic event. Results shown represent the mean ± SD of three groups of five animals each.

5.3. General procedure for the preparation of 6-methyl-benzo[1,3]dioxole-5-sulfonic acid-formyl-phenyl ester derivatives (**9a–c**)

To a solution of 1.45 mmol of sulfonyl chloride derivative (**8**) in 10 mL of methylene chloride, were added 1.45 mmol of the appropriate hydroxybenzaldehydes and 1.54 mmol of triethylamine. The reaction mixture was stirred for 2 h at room temperature, when the end of reaction was observed by TLC. The products (**9a–c**) were isolated by addition of 40 mL of methylene chloride and extraction with 10% aq. HCl (4 × 20 mL) and brine (3 × 20 mL). The organic layer was dried over anhydrous sodium sulfate, filtered and evaporated under reduced pressure to furnish the aldehyde derivatives (**9a–c**) in excellent yields.

5.3.1. 6-Methyl-benzo[1,3]dioxole-5-sulfonic acid 3-formyl-phenyl ester (**9a**)

Derivative **9a** was obtained as brown solid in 98% yield, mp 65–66 °C. ^{1}H NMR (200 MHz, $CDCl_3$) δ: 2.68 (s, ArCH_3); 6.04 (s, OCH_2O); 6.83 (s, H2); 7.22 (s, H5); 7.33–7.35 (m, H5′); 7.47–7.51 (d, $J = 8.1$ Hz, H6′); 7.50 (s, H2′); 7.78 (d, $J = 7.8$ Hz, H4′), 9.94 (s, ArCO*H*) ppm. ^{13}C NMR (50 MHz, $CDCl_3$) δ: 20.69 (Ar*C*H$_3$); 102.64 (O*C*H$_2$O); 110.49 (C5); 112.38 (C2); 122.77 (C2′); 126.08 (C3′); 128.29 (C6′); 128.50 (C5′); 130.64 (C4′); 135.46 (C1); 138.03 (C6); 146.04 (C4); 150.21 (C1′); 152.68 (C3); 190.75 (Ar*C*OH) ppm.

5.3.2. 6-Methyl-benzo[1,3]dioxole-5-sulfonic acid 4-formyl-phenyl ester (**9b**)

Derivative **9b** was obtained as yellow solid in 89% yield, mp 104–106 °C. ^{1}H NMR (200 MHz, $CDCl_3$) δ: 2.58 (s, ArCH_3); 5.95 (s, OCH_2O); 6.73 (s, H2); 7.12 (d, $J = 8.6$ Hz, H2′ and H6′); 7.15 (s, H5); 7.75 (d, $J = 8.6$ Hz, H3′ and H5′); 9.87 (s, ArCO*H*) ppm. ^{13}C NMR (50 MHz, $CDCl_3$) δ: 20.71 (Ar*C*H$_3$); 102.70 (O*C*H$_2$O); 110.55 (C5); 112.40 (C2); 122.93 (C2′ and C6′); 126.27 (C4′); 131.50 (C3′ and C5′); 134.97 (C1); 135.53 (C6); 146.11 (C4); 152.77 (C3); 154.01 (C1′); 190.78 (Ar*C*OH) ppm.

5.3.3. 6-Methyl-benzo[1,3]dioxole-5-sulfonic acid 2-formyl-phenyl ester (**9c**)

Derivative **9c** was obtained as brown solid in 81% yield, mp 98–100 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 2.66 (s, ArCH_3); 6.09 (s, OCH_2O); 6.85 (s, H2); 7.09 (d, $J = 8.1$ Hz, H6′); 7.29 (s, H5); 7.30 (dt, $J = 7.1$ Hz and 8.1 Hz, H5′); 7.58 (dt, $J = 7.1$ Hz and 7.7 Hz, H4′); 7.95 (d, $J = 7.7$ Hz, H3′); 10.20 (s, ArCO*H*) ppm. ^{13}C NMR (75 MHz, $CDCl_3$) δ: 20.80 (Ar*C*H$_3$); 102.70 (O*C*H$_2$O); 110.40 (C5); 112.60 (C2); 123.48 (C6′); 125.78 (C2′); 127.67 (C1); 128.89 (C4′); 129.74 (C3′); 135.46 (C5′); 135.78 (C6); 146.24 (C4); 151.38 (C1′); 152.98 (C3); 187.85 (Ar*C*OH) ppm.

5.4. General procedure for the preparation of (6-methyl-benzo[1,3]dioxole-5-sulfonyloxy)-benzoic acid methyl ester derivatives (**10a–c**)

To a mixture of 0.81 mmol of aldehyde derivatives (**9a–c**) in methanol (30 mL) was added 4.1 mmol of sodium cyanide and 12.2 mmol of activated manganese dioxide. The reactants were stirred at room temperature for 24 h and the suspension was filtered through Celite® and treated with dichloromethane (3 × 30 mL) and water (100 mL). The organic layers were joined, dried over anhydrous sodium sulfate, filtered and evaporated under reduced pressure to furnish methyl esters **10a–c** in good yields.

5.4.1. 3-(6-Methyl-benzo[1,3]dioxole-5-sulfonyloxy)-benzoic acid methyl ester (**10a**)

Derivative **10a** was obtained as yellow solid in 97% yield, mp 178–180 °C. ^{1}H NMR (200 MHz, $CDCl_3$) δ: 2.58 (s, ArCH_3); 3.80 (s, ArCO$_2$CH_3); 5.94 (s, OCH_2O); 6.73 (s, H2); 7.14 (s, H5); 7.15 (d, $J = 8.1$ Hz, H6′); 7.29 (dt, $J = 8.1$ Hz and 7.8 Hz, H5′); 7.58 (s, H2′); 7.84 (d, $J = 7.8$ Hz, H4′) ppm. ^{13}C NMR (50 MHz, $CDCl_3$) δ: 20.74 (Ar*C*H$_3$); 52.56 (s, ArCO$_2$*C*H$_3$); 102.60 (O*C*H$_2$O); 110.63 (C5); 112.35 (C2); 123.46 (C2′); 126.38 (C6′); 126.79 (C5′); 128.32 (C4′); 129.89 (C3′); 132.18 (C1); 135.48 (C6); 146.04 (C4); 149.64 (C1′); 152.61 (C3); 165.83 (Ar*C*O$_2$CH$_3$) ppm.

5.4.2. 4-(6-Methyl-benzo[1,3]dioxole-5-sulfonyloxy)benzoic acid methyl ester (**10b**)

Derivative **10b** was obtained as yellow solid in 80% yield, mp 99–101 °C. ^{1}H NMR (200 MHz, $CDCl_3$) δ: 2.66 (s, ArCH_3); 3.88 (s, ArCO$_2$CH_3); 6.04 (s, OCH_2O); 6.80 (s, H2); 7.09 (d, $J = 8.6$ Hz, H2′ and H6′); 7.22 (s, H5); 7.99 (d, $J = 8.6$ Hz, H3′ and H5′) ppm. ^{13}C NMR (50 MHz, $CDCl_3$) δ: 20.70 (Ar*C*H$_3$); 52.48 (ArCO$_2$CH$_3$); 102.64 (O*C*H$_2$O); 110.60 (C5); 112.32 (C2); 122.18 (C2′ and C6′); 126.29 (C4′); 129.04 (C1); 131.52 (C3′ and C5′); 135.45 (C6); 146.04 (C4); 152.65 (C3); 153.06 (C1′); 166.11 (Ar*C*O$_2$CH$_3$) ppm.

*5.4.3. 2-(6-Methyl-benzo[1,3]dioxole-5-sulfonyloxy)benzoic acid methyl ester (**10c**)*

Derivative **10c** was obtained as yellow solid in 60% yield, mp 106–108 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 2.64 (s, ArCH_3); 3.87 (s, ArCO_2CH_3); 6.04 (s, OCH_2O); 6.81 (s, H2); 6.92 (d, $J = 8.1$ Hz, H6′); 7.21 (s, H5); 7.32 (dt, $J = 6.3$ Hz and 7.6 Hz, H4′); 7.34 (dt, $J = 8.1$ Hz and 6.3 Hz, H5′); 7.89 (d, $J = 7.6$ Hz, H3′) ppm. ^{13}C NMR (75 MHz, $CDCl_3$) δ: 20.84 (ArCH_3); 52.59 (s, ArCO_2CH_3); 102.56 (OCH_2O); 110.40 (C5); 112.31 (C2); 123.57 (C6′); 126.07 (C2′); 127.17 (C1); 127.17 (C4′); 132.12 (C3′); 133.34 (C5′); 135.60 (C6); 145.91 (C4); 147.97 (C1′); 152.43 (C3); 165.46 (ArCO_2CH_3) ppm.

*5.5. General procedure for the preparation of 6-methyl-benzo[1,3]dioxole-5-sulfonic acid-hydrazinocarbonyl-phenyl ester derivatives (**11a–c**)*

A solution of 1.14 mmol of the appropriate methyl ester derivatives (**10a–c**) and 6 mL of 80% hydrazine hydrate in 15 mL of ethanol, was stirred at room temperature for 2 h, when the end of reaction was observed by TLC. Then, the mixture was cooled and neutralized with concentrated HCl, resulting in the formation of a white precipitate, which was filtered, washed with water and dried under reduced pressure to give hydrazide derivatives (**11a–c**) in good yields.

*5.5.1. 6-Methyl-benzo[1,3]dioxole-5-sulfonic acid-3-hydrazinocarbonyl-phenyl ester derivatives (**11a**)*

Derivative **11a** was obtained as white solid in 78% yield, mp 166–168 °C. IR (KBr): 3343 and 3279 (N–H), 1673 (C=O), 1350 (S=O) cm^{-1}.

*5.5.2. 6-Methyl-benzo[1,3]dioxole-5-sulfonic acid-4-hydrazinocarbonyl-phenyl ester derivatives (**11b**)*

Derivative **11b** was obtained as white solid in 81% yield, mp 130–131 °C. IR (KBr): 3348 and 3279 (N–H), 1668 (C=O), 1357 (S=O) cm^{-1}.

*5.5.3. 6-Methyl-benzo[1,3]dioxole-5-sulfonic acid-2-hydrazinocarbonyl-phenyl ester derivatives (**11c**)*

Derivative **11c** was obtained as white solid in 75% yield, mp 128–130 °C. IR (KBr): 3340 and 3275 (N–H), 1669 (C=O), 1355 (S=O) cm^{-1}.

*5.6. General procedure for the preparation of 6-methyl-benzo[1,3]dioxole-5-sulfonic acid-benzylidene-hydrazinocarbonyl-phenyl ester derivatives (**4b–j**)*

An equimolar amount of appropriate aromatic aldehyde was added to a solution of hydrazide derivatives **11a–c** (1.05 mmol) in 20 mL of ethanol, in the presence of catalytic amount of hydrochloric acid. The reaction was stirred for 0.5–1.0 h, at reflux, and the solvent was evaporated under reduced pressure. The colored precipitate was collected by filtration, washed with cold water and dried under vacuum to give the desired *N*-acylhydrazone derivatives (**4b–j**), that were purified by recrystallization in ethanol, yielding compounds **4b–j** in excellent yields.

*5.6.1. 6-Methyl-benzo[1,3]dioxole-5-sulfonic acid 3-(pyridin-4-ylmethylene-hydrazinocarbonyl)-phenyl ester (**4b**)*

Derivative **4b** was obtained as a white solid by condensation of **11a** with pyridine-4-carboxaldehyde in 89% yield. ^{1}H NMR (200 MHz, DMSO-d_6) δ: 2.55 (s, ArCH_3); 6.17 (s, OCH_2O); 7.20 (d, $J = 7.9$ Hz, H6′); 7.22 (s, H2); 7.29 (s, H5); 7.58 (dt, $J = 7.7$ Hz and 7.9 Hz, H5′); 7.69 (s, H2′); 7.94 (m, H4′, H2″ and H6″); 8.54 (s, C*H*=N); 8.77 (m, $J = 7.9$ Hz, H3″ and H5″); 12.58 (br, CON*H*) ppm. ^{13}C NMR (50 MHz, DMSO-d_6) δ: 19.87 (ArCH_3); 102.90 (OCH_2O); 109.39 (C5); 112.44 (C2); 121.52 (C2′); 121,99 (C6′); 124.92 (C4′); 125.51 (C6″); 126.76 (C2″); 130.45 (C5′); 134.67 (C3′); 135.09 (C1); 135.61 (C6); 144.26 (C1″); 145.08 (*C*H=N); 147.64 (C3″ and C5″); 145.76 (C4); 148.95 (C1′); 152.52 (C3); 162.56 (Ar*C*ONH) ppm.

*5.6.2. 6-Methyl-benzo[1,3]dioxole-5-sulfonic acid 3-(benzylidene-hydrazinocarbonyl)-phenyl ester (**4c**)*

Derivative **4c** was obtained as a white solid by condensation of **11a** with benzaldehyde in 84% of yield. ^{1}H NMR (200 MHz, DMSO-d_6) δ: 2.61 (s, ArCH_3); 6.15 (s, OCH_2O); 7.08–7.16 (m, H2, H5 and H6′); 7.45–7.49 (m, H2′, H4′ and H5′); 7.82–7.89 (m, H2″, H3″, H4″, H5″ and H6″); 8.45 (s, C*H*=N); 11.90 (br, CON*H*) ppm. ^{13}C NMR (50 MHz, DMSO-d_6) δ: 20.34 (ArCH_3); 103.37 (OCH_2O); 109.89 (C5); 112.91 (C2); 121.76 (C2′); 125.45 (C6′); 126.96 (C4′); 127.67 (C3″ and C5″); 129.34 (C2″ and 6″); 130.73 (C5′); 130.85 (C4″); 134.62 (C3′); 135.55 (C1″); 135.72 (C1); 136.25 (C6); 148.99 (C4); 149.00 (*C*H=N); 149.45 (C1′); 152.99 (C3); 162.03 (Ar*C*ONH) ppm.

*5.6.3. 6-Methyl-benzo[1,3]dioxole-5-sulfonic acid 3-(4-nitro-benzylidene-hydrazinocarbonyl)-phenyl ester (**4d**)*

Derivative **4d** was obtained as a yellow solid by condensation of **11a** with 4-nitrobenzaldehyde in 98% of yield. ^{1}H NMR (300 MHz, DMSO-d_6) δ: 2.67 (s, ArCH_3); 6.04 (s, OCH_2O); 6.82 (s, H2); 7.13 (d, $J = 7.4$ Hz, H6′); 7.22 (s, H2′); 7.26 (s, H5); 7.40 (m, H5′); 7.65 (m, H4′); 7,85 (d, $J = 7.6$ Hz, H2″ and H6″); 8.22 (d, $J = 7.6$ Hz, H3″ and H5″); 8.31 (s, C*H*=N); 11.65 (br, CON*H*) ppm. NMR ^{13}C (75 MHz, DMSO-d_6) δ: 20.39 (ArCH_3); 103.25 (OCH_2O); 109.89 (C5); 112.90 (C2); 121.60 (C2′); 121.83 (C1″); 123.75 (C3″ and C5″); 125.21 (C6′); 125.61 (C5′); 126.84 (C4′); 129.49 (C2″ and C6″); 130.68 (C3′), 135.59 (C1); 136.20 (C6); 146.29 (C4); 149.38 (*C*H=N); 150.21 (C1′); 150.85 (C4″); 153.10 (C3); 161.53 (Ar*C*ONH) ppm.

*5.6.4. 6-Methyl-benzo[1,3]dioxole-5-sulfonic acid 3-(4-cyano-benzylidene-hydrazinocarbonyl)-phenyl ester (**4e**)*

Derivative **4e** was obtained as a white solid by condensation of **11a** with 4-cyanobenzaldehyde in 94% of yield. ^{1}H NMR (200 MHz, DMSO-d_6) δ: 2.60 (s, ArCH_3); 6.14 (s,

OCH_2O); 7.18 (d, $J = 8.8$ Hz, H6′); 7.20 (s, H2); 7.26 (s, H5); 7.56 (dt, $J = 8.1$ Hz and 7.8 Hz, H5′); 7.64 (s, H2′); 7.86–7.94 (m, H4′, H2″, H6″, H3″ and H5″); 8.48 (s, C*H*=N); 12.17 (br, CON*H*) ppm. ^{13}C NMR (50 MHz, DMSO-d_6) δ: 20.38 (Ar*C*H$_3$); 103.42 (O*C*H$_2$O); 109.93 (C5); 112.60 (C4″); 112.96 (C2); 119.15 (Ar*C*N); 121.90 (C2′); 125.49 (C6′); 125.79 (C5′); 127.14 (C4′); 128.25 (C2″ and C6″); 130.68 (C3′), 133.28 (C3″ and C5″); 135.45 (C1″); 135.61 (C1); 136.12 (C6); 146.29 (C4); 147.06 (*C*H=N); 149.50 (C1′); 153.05 (C3); 162.35 (Ar*C*ONH) ppm.

5.6.5. 6-Methyl-benzo[1,3]dioxole-5-sulfonic acid 3-(4-carboxy-benzylidene-hydrazinocarbonyl)-phenyl ester (**4f**)

Derivative **4f** was obtained as a white solid by condensation of **11a** with 4-carboxybenzaldehyde in 91% yield. ^{1}H NMR (200 MHz, DMSO-d_6) δ: 2.50 (s, ArC*H*$_3$); 6.04 (s, OC*H*$_2$O); 7.18 (s, H2); 7.20 (s, H5); 7.22 (d, $J = 7.9$ Hz, H6′); 7.56 (dt, $J = 8.1$ Hz and 7.9 Hz, H5′); 7.62 (s, H2′); 7.84 (d, $J = 8.3$ Hz, H2″ and H6″); 7,86 (d, $J = 8.1$ Hz, H4′); 8.01 (d, $J = 8.3$ Hz, H3″ and H5″); 8.49 (s, C*H*=N); 11.95 (br, CON*H*); 12.05 (br, COO*H*) ppm. ^{13}C NMR (50 MHz, DMSO-d_6) δ: 20.35 (Ar*C*H$_3$); 103.38 (O*C*H$_2$O); 109.89 (C5); 112.93 (C2); 121.83 (C2′); 125.48 (C6′); 125.81 (C5′); 127.04 (C4′); 127.68 (C2″ and C6″); 129.01 (C3′), 130.29 (C3″ and C5″); 132,36 (C4″); 135.58 (C1); 135.61 (C1″); 138.66 (C6); 146.27 (C4); 147.82 (*C*H=N); 149.46 (C1′); 153.01 (C3); 162.23 (Ar*C*ONH); 167.38 (Ar*C*OOH) ppm.

5.6.6. 6-Methyl-benzo[1,3]dioxole-5-sulfonic acid 3-[(pyridine-4-carbonyl)-hydrazonomethyl]-phenyl ester (**4h**)

Derivative **4h** was obtained as a white solid by condensation of aldehyde derivative **9a** with isonicotinic acid hydrazide in 78% yield. ^{1}H NMR (200 MHz, DMSO-d_6) δ: 2.50 (s, ArC*H*$_3$); 2.56 (ArC*H*$_3$); 6.12 (s, OC*H*$_2$O); 7.04 (d, $J = 7.2$ Hz, H6′); 7.11 (s, H2); 7.16 (s, H5); 7.42 (d, $J = 8.9$ Hz, H2″ and H6″); 7.64 (m, H4′); 7.94 (s, C*H*=N); 8.01 (m, H2′ and H5′); 8.51 (s, C*H*=N); 8.9 (m, H3″ and H5″); 12.37 (br, CON*H*); 12.53 (br, CON*H*) ppm. ^{13}C NMR (50 MHz, DMSO-d_6) δ: 20.35 (Ar*C*H$_3$); 103.38 (O*C*H$_2$O); 109.86 (C5); 112.90 (C2); 120.42 (C2′); 123,48 (C6′); 124.00 (C2″ and C6″); 125.54 (C4′); 127.01 (C5′); 131.21 (C3′); 135.52 (C6); 136.59 (C1); 143.45 (C1″); 146.11 (*C*H=N); 146.27 (C4); 148.18 (*C*H=N); 148.40 (C3″ and C5″); 149.83 (C1′); 152.99 (C3); 161.38 (Ar*C*ONH) ppm.

5.6.7. 6-Methyl-benzo[1,3]dioxole-5-sulfonic acid 4-(4-carboxy-benzylidene-hydrazinocarbonyl)-phenyl ester (**4i**)

Derivative **4i** was obtained as a white solid by condensation of **11b** with 4-carboxybenzaldehyde in 89% yield. ^{1}H NMR (200 MHz, DMSO-d_6) δ: 2.61 (s, ArC*H*$_3$); 6.16 (s, OC*H*$_2$O); 7.16 (s, H2); 7.21 (s, H5); 7.23 (d, $J = 7.8$ Hz, H2′ and H6′); 7.83 (d, $J = 8.1$ Hz, H2″ and H6″); 7.92 (d, $J = 7.8$ Hz, H3′ and H5′); 8.0 (d, $J = 8.1$ Hz, H3″ and H5″); 8.46 (s, C*H*=N); 12.05 (s, ArCON*H*) ppm. ^{13}C NMR (50 MHz, DMSO-d_6) δ: 20.41 (Ar*C*H$_3$); 103.49 (O*C*H$_2$O); 110.02 (C5); 113.03 (C2); 122.47 (C2′ and C6′); 125.54 (C4′); 127.72 (C3′ and C5′); 130.36 (C2″, C3″, C5″ and C6″); 132.36 (C1); 132.85 (C4″); 135.68 (C6); 138.80 (C1″); 147.45 (*C*H=N); 147.46 (C4); 151.85 (C3); 153.11 (C1′); 162.78 (Ar*C*ONH); 167.46 (Ar*C*O$_2$H) ppm.

5.6.8. 6-Methyl-benzo[1,3]dioxole-5-sulfonic acid 2-(4-carboxy-benzylidene-hydrazinocarbonyl)-phenyl ester (**4j**)

Derivative **4j** was obtained as a white solid by condensation of **11c** with 4-carboxybenzaldehyde in 89% yield. ^{1}H NMR (200 MHz, DMSO-d_6) δ: 2.61 (s, ArC*H*$_3$); 6.15 (s, OC*H*$_2$O); 7.14 (s, H2); 7.21 (s, H5); 7.23 (d, $J = 8.5$ Hz, H6′); 7.85–8.03 (m, H3′, H4′, H5′, H2″, H3″, H5″ and H6″); 8.47 (s, C*H*=N); 12.06 (s, RCO$_2$*H*) ppm. ^{13}C NMR (50 MHz, DMSO-d_6) δ: 20.37 (Ar*C*H$_3$); 103.45 (O*C*H$_2$O); 109.99 (C5); 112.98 (C2); 121.84 (C6′ and C4′); 125.48 (C2′); 127.68 (C1 and C3′); 130.32 (C2″, C6″, C3″ and C5″); 132.31 (C4″); 132.79 (C1″); 135.64 (C5′); 138.76 (C6); 146.28 (C4); 147.43 (*C*H=N); 151.82 (C3); 153.06 (C1′);162.77 (Ar*C*ONH); 167.43 (Ar*C*O$_2$H) ppm.

5.7. 6-Methyl-benzo[1,3]dioxole-5-sulfonic acid 3-(4-amino-benzylidene-hydrazino carbonyl)-phenyl ester (**4a**)

A mixture of nitro-acylhydrazone **4d** (0.41 mmol), iron powder (0.123 g) and ammonium chloride (0.034 g; 0.24 mmol) in 15 mL of a mixture of ethanol and water (2:1) was refluxed for 30 min. Then, the reaction mixture was filtered hot, and the filtrate was evaporated to half of its original volume, followed by addition of 20 mL of water and extraction with AcOEt (3 × 20 mL). The organic layer was dried (Na_2SO_4), filtered and evaporated under reduced pressure to give the desired amine-derivative in 75% yield. ^{1}H NMR (300 MHz, DMSO-d_6) δ: 2.60 (s, ArC*H*$_3$); 5.63 (br, ArN*H*2); 6.15 (s, OC*H*$_2$O); 6.60 (d, $J = 8.4$ Hz, H3″ and H5″); 7.2 (m, H2 and H5 and H6′); 7.40 (d, $J = 8.4$ Hz, H2″ and H6″); 7,53 (dt, $J = 8.1$ Hz and 8.0 Hz, H5′); 7.60 (s, H2′); 7.85 (d, $J = 8.1$ Hz, H4′); 8.31 (s, C*H*=N); 11.58 (br, CON*H*) ppm. ^{13}C NMR (75 MHz, DMSO-d_6) δ: 20.38 (Ar*C*H$_3$); 103.40 (O*C*H$_2$O); 109.93 (C5); 112.95 (C2); 114.12 (C3″ and C5″); 121.62 (C2′); 121.80 (C1″); 125.21 (C6′); 125.49 (C5′); 126.83 (C4′); 129.33 (C2″ and C6″); 130.78 (C3′), 135.58 (C1); 136.17 (C6); 146.28 (C4); 149.46 (*C*H=N); 150.16 (C1′); 151.68 (C4″); 153.02 (C3); 161.49 (Ar*C*ONH) ppm.

5.8. 6-Methyl-benzo[1,3]dioxol-5-sulfonic acid 3-[4-(N-hydroxycarbamimidoyl)-benzylidene-hydrazino carbonyl]-phenyl ester (**4g**)

Hydroxylamine hydrochloride (0.97 mmol) was added to a solution of cyano-acylhydrazone **4e** (0.32 mmol) and Na_2CO_3 (1.3 mmol) in ethanol (10 mL) and H_2O (5 mL). The mixture was heated at reflux temperature for 8 h, then, it was cooled and neutralized with concentrated HCl, followed by extraction with AcOEt (3 × 20 mL). The organic layer was dried (Na_2SO_4), filtered and evaporated under reduced pressure. The crude material was purified by column

chromatography on silica gel (CH_2Cl_2/MeOH 2% and 5%) (yield: 69%). ^{1}H NMR (200 MHz, DMSO-d_6) δ: 2.49 (s, ArCH_3); 3.49 (br, NH_2); 5.95 (s, OCH_2O); 7.1 (s, H2); 7.18 (s, H5); 7.25 (d, $J = 7.9$ Hz, H6′); 7.56 (dt, $J = 8.1$ Hz and 7.9 Hz, H5′); 7.65 (s, H2′); 7,85 (d, $J = 8.1$ Hz, H4′); 7.87 (d, $J = 8.0$ Hz, H2″ and H6″); 7.89 (d, $J = 8.0$ Hz, H3″ and H5″); 8.14 (s, CH=N); 9.9 (br, OH); 11.95 (br, CONH) ppm. ^{13}C NMR (50 MHz, DMSO-d_6) δ: 20.39 (ArCH$_3$); 103.43 (OCH$_2$O); 109.99 (C5); 112.95 (C2); 122.93 (C2′); 125.52 (C6′); 125.64 (C5′); 127.90 (C4′); 128.47 (C2″ and C6″); 130.10 (C3′), 130.82 (C3″ and C5″); 135,23 (C4″); 135.61 (C1); 136.26 (C1″); 146.30 (C6); 148.19 (C4); 148.36 (CH=N); 153.05 (C1′); 157.96 (C3); 168.07 (ArCONH); 168.67 (ArC=(N−OH)NH$_2$).

5.9. Molecular modeling

The conformer distribution of 6-methyl-benzo[1,3]dioxole-5-sulfonic acid (4-carboxy-benzylidene-hydrazinocarbonyl)-phenyl ester derivatives (**4f**, **4i**, **4j**) was carried out using SYBYL molecular mechanics. The geometry optimization was performed using the semiempirical AM1 Hamiltonian [21] with SPARTAN 1.0.5 program [22] on Pentium IV 1.5 GHz.

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