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Flavonoids with an Oligopolysulfated Moiety: A New Class of Anticoagulant Agents

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Polysulfated (oligo)flavonoids were synthesized and assayed for their in vitro and in vivo anticoagulant activities. The approach was based on molecular hybridization of two classes of anticoagulants, sulfated polysaccharides and sulfated flavonoids. The synthesis was optimized using microwave-assisted sulfation with triethylamine-sulfur trioxide. The obtained polysulfated flavonosides were highly effective in increasing clotting times and able to completely block the clotting process, in contrast to their corresponding aglycones. The thromboelastography proved that polysulfated flavonosides possess good whole blood anticoagulation activity. The following structure−activity relationships were found: 3-*O*-rutinosides (**10**, **13**) were direct inhibitors of FXa, while 7-*O*-rutinosides (**7**, **8**) showed inhibition of FXa by ATIII activation. Furthermore, compounds **7** and **13** were stable in plasma and active in vivo and preliminary toxicity studies would lead us to rule out acute side effects. From the overall results, the polysulfated flavonosides showed the potential as new effective and safe agents for anticoagulant therapy.

Introduction

Heparin is a widespread anticoagulant agent,[1,2] exerting its therapeutic effect by binding the enzyme inhibitor antithrombin III (ATIII[a]), which results in the inactivation of factor Xa (FXa) and thrombin.[3] Heparin is obtained from either bovine or porcine sources, thus making heparin preparations structurally diverse, varying in their molecular weight, monosaccharide sequence, and sulfation pattern.[4,5] The use of heparin suffers from a number of limitations due to its polyanionic and heterogeneous nature. Heparin therapy is limited to intravenous application[6] and can be associated to cases of excessive bleeding[7] and liver toxicity.[8] Furthermore, a significant number of patients develop heparin-induced thrombocytopenia (HIT) after prolonged heparin exposure.[9] Some problems associated with heparin were minimized with the preparation of low-molecular-weight heparins (LMWH),[10,11] which induce less bleeding complications.[12] Because of the smaller molecular size, bioavailability was improved, allowing subcutaneous administration.[13] However, like heparin, these derivatives are still heterogeneous and polydisperse[14,15] and the risk of inducing the immunological type of HIT has also been reported.[16] The structural features associated with HIT have been characterized[17] and a heparin pentasaccharide, fondaparinux (Figure 1), completely free of HIT activation, was developed and approved.[18,19] In contrast to unfractionated and LMW heparins, fondaparinux has a defined composition.[20] However, the synthesis of this pentasaccharide is accomplished through a multistep, complex, and low-yielding procedure.[21] Therefore, the search for new alternative anticoagulant agents with a well-defined composition, fewer secondary effects, and feasible synthesis is a major challenge to medicinal chemists.

Our top priority was to develop new anticoagulant small molecules based on a flavonoid scaffold. Several sulfated flavonoids have already been described for their anticoagulant actions.[22] Two sulfated flavonoids isolated from *Flaveria bidentis,* quercetin 3-acetyl-3′,4′,7-*O*-trisulfate and quercetin 3,3′,4′,7-*O*-tetrasulfate (Figure 1), have already shown anticoagulant actions in vitro by prolonging activated partial thromboplastin (APTT) and prothrombin (PT) times through a heparin cofactor II (HCII)-dependent thrombin inhibition.[23] Moreover, quercetin 3,3′,4′,7-*O*-tetrasulfate was found to inhibit agonist-induced human platelet aggregation.[24] Also, the inhibitory effects of quercetin 7-*O*-sulfate and quercetin 4′,7-*O*-disulfate sodium salts (Figure 1) on thrombin-induced platelet aggregation through a mechanism involving Ca^{2+} influx inhibition were reported.[25,26] Several sulfated flavonoids obtained by synthesis were shown to act as activators of ATIII/FXa inhibition.[27−30] However, the activation effect achieved with these sulfated small molecules on ATIII was weak.[27−30] As assessed by a

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[a] Abbreviations: ATIII, antithrombin III; FXa, factor Xa; HIT, heparin-induced thrombocytopenia; LMWH, low-molecular-weight heparins; HCII, heparin cofactor II; APTT, activated partial thromboplastin time; PT, prothrombin time; EHBS, extended heparin binding site; SOSA, selective optimization of side activities; DMA, dimethylacetamide; MW, microwave; $APTT_2$, concentration of compound required to double the time of clot formation in the APTT assay; PT_2, concentration of compound required to double the time of clot formation in the PT assay; TT, thrombin time; TT_2, concentration of compound required to double the time of clot formation in the TT assay; TEG, thromboelastography; PFA, platelet function analyzer; ip, intraperitoneal; ADP, adenosine 5′-diphosphate; EPI, epinephrine; GOT, glutamic-oxaloacetic transaminase; GPT, glutamate-pyruvate-transaminase; SEM, standard error of the mean.

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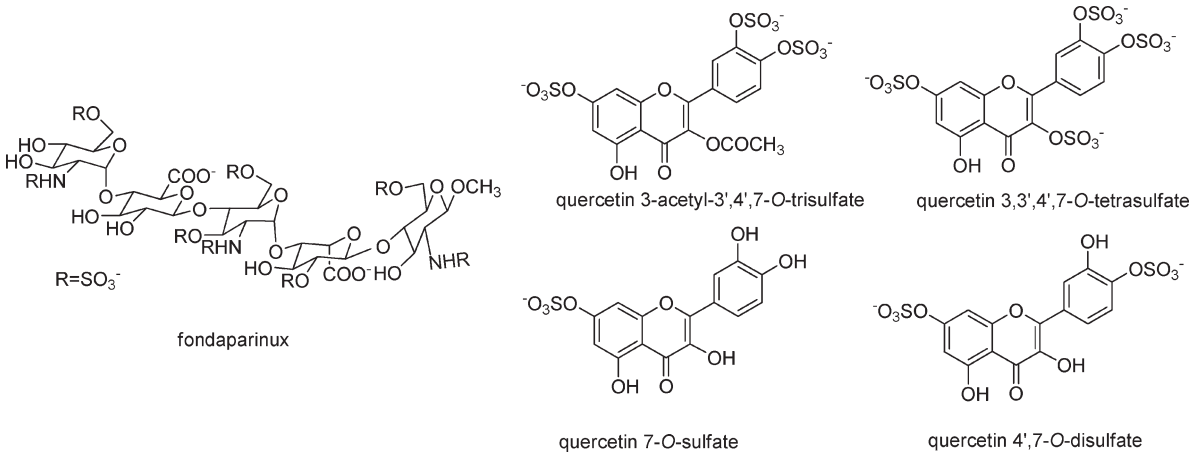


Figure 1. Pentasaccharide fondaparinux and flavonoids with anticoagulant activity.

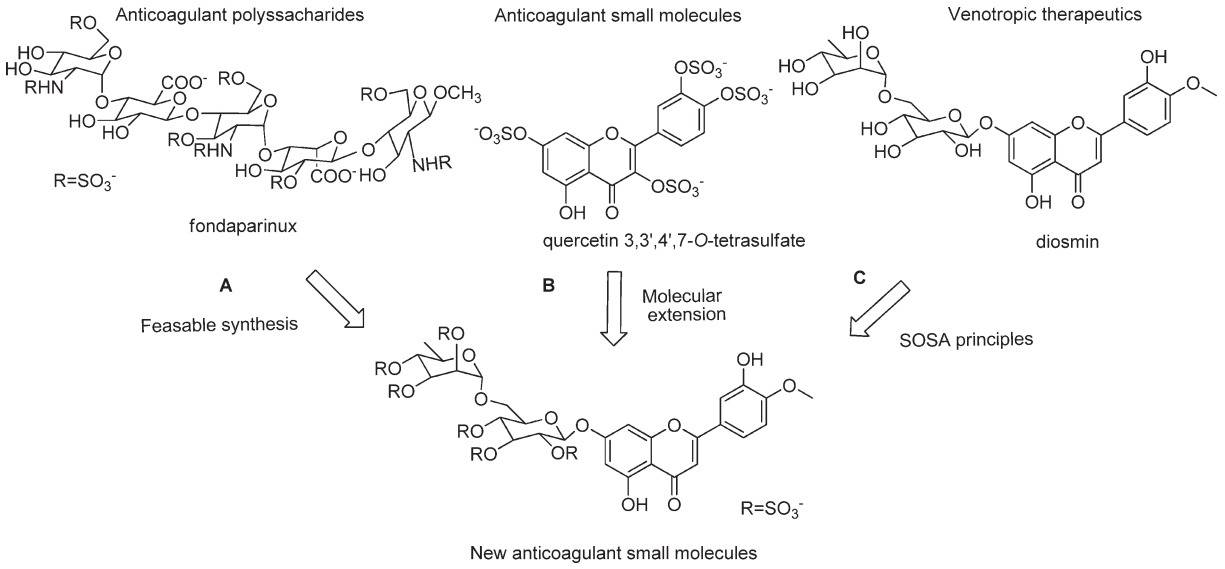


Figure 2. Schematic representation of the strategies used herein for the development of new anticoagulant small molecules based on three examples, namely fondaparinux, quercetin 3,3′,4′,7-*O*-tetrasulfate, and diosmin.

computational study based on the hydropathic interaction (HINT) analysis, the increase of the flavonoid molecular size was associated to an improvement of the binding affinity toward ATIII. For instance, a dimeric compared to a monomeric molecule scored better because it could simultaneously engage both the extended heparin (EHBS) and pentasaccharide (PBS) binding sites of ATIII.[29]

The oligo-*O*-sulfated moiety is present in potent anticoagulant polysaccharides, suggesting the incorporation of an oligo-*O*-sulfated moiety into a flavonoid scaffold in order to improve the anticoagulant potency by increasing both molecular size and number of sulfate groups (Figure 2, A and B). Modification of molecules that have already been safely used in humans conducts to compounds with more predictable and less complex pharmacokinetics, lower incidence of side effects, and less demanding clinical studies.[31] On the basis of these considerations, we selected for sulfation several flavonoids glycosides already used in human therapy as venotropic agents[32] (Figure 2C). This strategy follows the principles of SOSA (selective optimization of side activities) approach.[31]

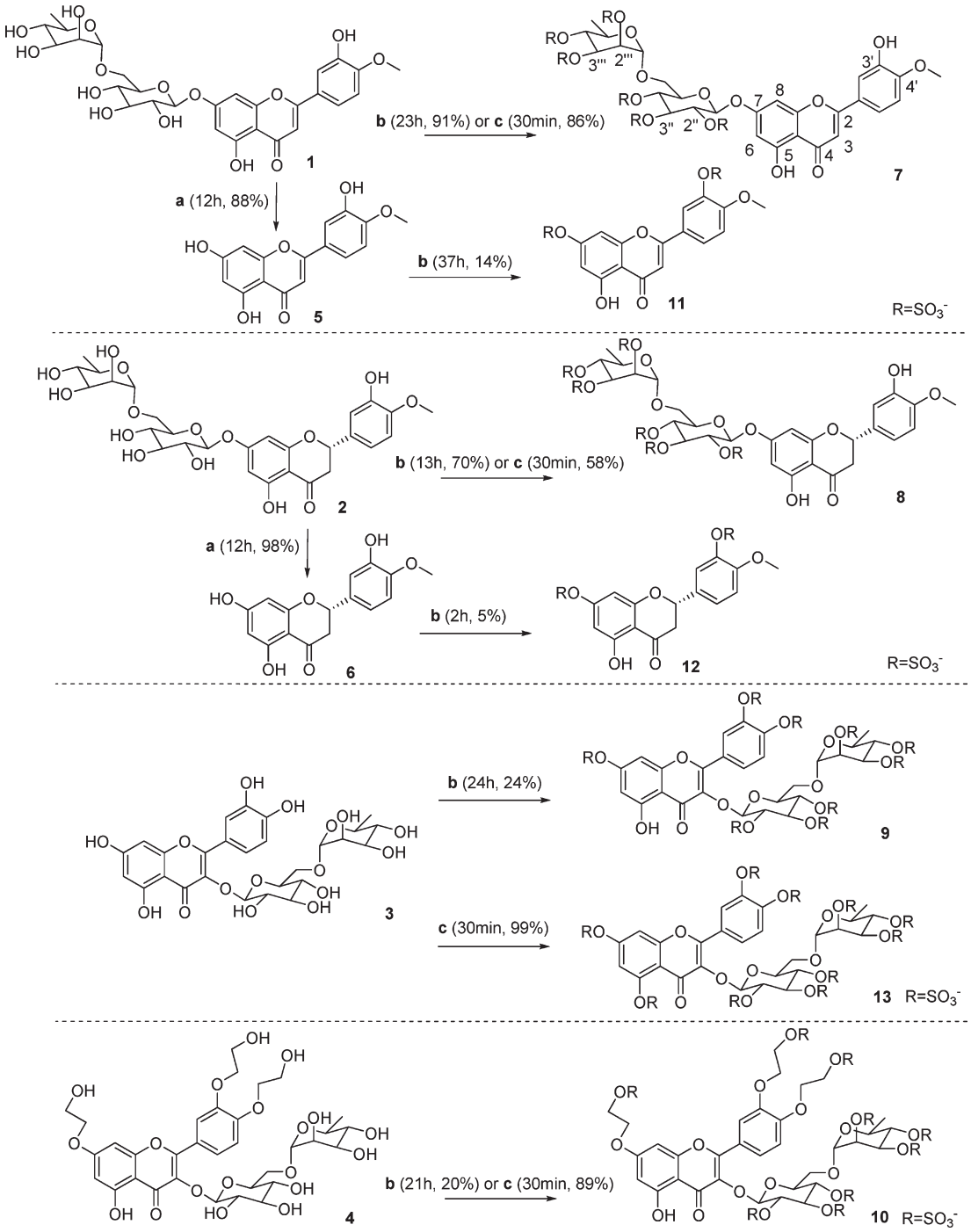
In this work, the synthesis, the in vitro and in vivo anticoagulant profile, and preliminary toxicological investigation of several polysulfated flavonoids/flavonosides are described. Compounds were screened by in vitro clotting time assays in human plasma, and the effect of the most promising molecules was further evaluated in human whole blood using thromboelastography and platelet function analyzer. To better understand the mechanism of action of these new anticoagulant molecules, in vitro enzyme inhibition assays were performed against FXa, thrombin, and ATIII. Finally, stability and in vivo studies were conducted for one representative direct and indirect inhibitor of FXa to determine in vivo anticoagulant activity and their toxicity potential.

Chemistry

Diosmin (**1**), hesperidin (**2**), rutin (**3**), and trihydroxyethylrutin (**4**) were selected as the building blocks for the synthesis of polysulfated flavonosides (Scheme 1). Additionally, in order to study structure−activity relationship, the respective aglycones diosmetin (**5**) and hesperetin (**6**) were submitted to sulfation (Scheme 1).

Flavonoids **5** and **6** were initially obtained by hydrolysis of **1** and **2**, respectively, in a sulfuric acid−acetic acid−water medium (Scheme 1, a).[33] Compounds **1**−**6** were then submitted to sulfation with triethylamine-sulfur trioxide adduct in

Scheme 1. Reagents and Conditions[a] for the Synthesis of Compounds **5**−**13**



[a] Reagents and conditions: (a) sulfuric acid−acetic acid−water (1:30:19); (b) triethylamine-sulfur trioxide adduct (4 equiv/OH for compounds **1**−**4**, **6**; 8 equiv/OH for compound **5**), DMA, 65 °C; (c) triethylamine-sulfur trioxide adduct (10 equiv/OH), DMA, MW.

dimethylacetamide (DMA) (Scheme 1, b). Sulfation was first attempted with tetrabuthylammonium hydrogen sulfate and *N*, *N*′-dicyclohexylcarbodiimide, a method extensively used in sulfation of flavonoids;[34] nonetheless, a complex mixture of sulfated derivatives was obtained (data not shown). The triethylamine−sulfur trioxide adduct in DMA (4−8 equiv/OH) allowed us to achieve a highly degree of sulfation.[28,30] Following conventional heating (Scheme 1, b), diosmin 2″,2‴,3″,3‴,4″,4‴-*O*-hexasulfate (**7**), hesperidin 2″,2‴,3″,3‴,4″,4‴-*O*-hexasulfate (**8**), rutin 2″,2‴,3′,3″,3‴,4′,4″,4‴,7-*O*-nonasulfate (**9**), 3″,4‴-*bis*(2-*O*-sulfate ethoxy)-7-(2-*O*-sulfate ethoxy)-rutin (**10**), diosmetin 3′,7-*O*-disulfate (**11**), and hesperetin 3′,7-*O*-disulfate (**12**) were obtained in moderate yields (Scheme 1). Compounds **7**, **8**, **10**, and **12** are described for the first time. Diosmetin 3′,7-*O*-disulfate (**11**) was previously isolated from the leaves of *Lachenalia unifolia*,[35] and rutin nonasulfate (**9**) was previously obtained by synthesis.[36]

The sulfation of the hydroxyl at 5-position did not occur in flavonoids **7**−**12**, which can be explained by the hydroxyl binding with the adjacent carbonyl at the 4-position. Surprisingly, for compounds **7** and **8**, sulfation did not take place in the 3′ hydroxyl, in contrast to compounds **11** and **12**. This fact

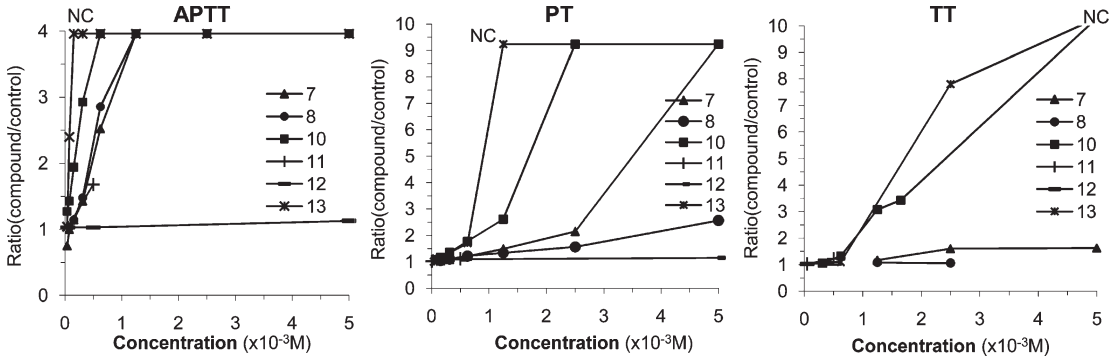


Figure 3. Effects of polysulfated flavonoids (**7**, **8**, **10**−**13**) on APTT, PT, and TT clotting assays using human pooled plasma, expressed as ratio of clotting time in the presence/absence of compound. Data points represent the average of three experiments performed in duplicate with a standard deviation < 10%. NC, no coagulation.

Table 1. Effects[a] of Polysulfated Flavonosides **7**, **8**, **10**, and **13** in the Clotting Assays

	activated partial thromboplastin time (APTT)		prothrombin time (PT)		thrombin time (TT)	
compd	total inhibition	$APTT_2$[b]	total inhibition	PT_2[b]	total inhibition	TT_2[b]
7	1.25	0.435	5	2.234		> 5
8	1.25	0.413		3.615		na
10	0.625	0.145	2.5	0.835	5	0.867
13	0.312	0.066	1.25	0.649	5	0.877

[a] Values in concentrations expressed in 10^{-3} M represent the average of three independent experiments with a standard deviation < 10%; positive control, heparin: $APTT_2$ = 0.4 U/mL, PT_2 = 1.9 U/mL, and TT_2 = 0.2 U/mL. [b] The concentration required to double clotting time. na: not active at 5×10^{-3} M ($P > 0.05$).

can be explained because the sulfation becomes progressively difficult, due to anion crowding, considering a large number of hydroxyl groups.[37]

The major bottleneck in the synthetic procedure was the isolation of the polysulfated compounds from the crude product. Because of the presence of an oligo-*O*-sulfated moiety, the flavonoids derivatives synthesized are highly soluble in water. To remove water-soluble impurities, dialysis with cellulose membrane (cutoff 1000) was necessary for compounds **8**−**10** (see Supporting Information).

To optimize the reaction, namely the time needed for sulfation of flavonoids glycosides with a high number of hydroxyl groups (**1**−**4**), new conditions were investigated by using microwave (MW) irradiation (Scheme 1, c). Recently, sulfation with MW was described using trimethyamine−sulfur trioxide in CH_3CN.[37] In the case of compounds **1**−**4**, DMA was found a more suitable solvent than CH_3CN. To avoid the presence of partially sulfated products, a 10 equiv/OH of the sulfating complex was used.[37]

When compared to conventional heating, the MW-assisted sulfation allowed the synthesis of highly sulfated derivatives in shorter reaction times (30 min). Additionally, rutin persulfate **13** was obtained (Scheme 1, c) in quantitative yield (99%) and with high purity (> 95%) in contrast to the previously described[38] as well as compound **10** with an increased yield (from 20 to 89%).

The structure elucidation of compounds **5**−**13** was established on the basis of IR, HRMS, and NMR techniques (see Supporting Information for detailed discussion). For aglycones **5** and **6**, data are in accordance to literature.[39,40] For diosmetin 3′,7-*O*-disulfate (**11**),[35] rutin nonasulfate (**9**),[36] and rutin decasulfate (**13**)[36] their structural data are reported for the first time in this work (see Experimental Section).

Results and Discussion

The anticoagulant activity of the sulfated molecules **7**, **8**, and **10**−**13** was initially screened in vitro in human plasma by APTT, PT, and thrombin time (TT). The three clotting assays recorded interactions at different stages of the coagulation process, providing key information about the mode of action. The APTT identifies the time interval required for clot formation in response to a nonphysiological stimulus that leads to primary activation of the intrinsic pathway; the PT assay measures the activity of coagulation factors of the extrinsic pathway, and the TT screens the formation of fibrin from fibrinogen after the addition of known amounts of thrombin to the plasma sample.[41] The anticoagulant effect of the desulfated starting materials (**2**−**4**) was also evaluated in order to explore their potential influence in the anticoagulant effect of the sulfated molecules. Because of solubility issues, only diosmin (**1**) was not considered because the final concentration of DMSO interfered with the clotting assays.

The results for the polysulfated flavonoids (**7**, **8**, **10**−**13**), on APTT, PT, and TT for the range of investigated concentrations are summarized in Figure 3. For the most potent compounds (**7**, **8**, **10**, and **13**), the concentration needed to double the coagulation times ($APTT_2$, PT_2, TT_2) was calculated (Table 1). Compound **11** was not tested at a final concentration of 5×10^{-3} M (not soluble), and compound **9** was not obtained with the desirable purity to be tested (< 95%).

The investigated sulfated compounds (**7**, **8**, **10**, and **13**) showed anticoagulant properties in a dose-dependent manner (Figure 3). The nonsulfated flavonoids **2**−**4** were inactive in all clotting time assays (at the concentration tested, 5×10^{-3} M, no significant difference was observed between the compound and the control; data not shown).

Prolongation of APTT (Figure 3) was observed for all the compounds (**7**, **8**, **10**−**13**). Flavonosides **7**, **8**, **10**, and **13** doubled APTT ($APTT_2$) at concentrations in the micromolar range and were able to completely block this clot formation pathway (Figure 3 and Table 1). The flavonoside **13** was the most potent in prolonging APTT ($APTT_2$ = 66 μM). The sulfated aglycone **11** prolonged no more than 1.65-fold the APTT at the highest

Table 2. Effects[a] of Polysulfated Flavonosides **7**, **8**, **10** (6.25×10^{-4} M), and **13** (1.25×10^{-4} M)[b] in Whole Human Blood on the Parameters Obtained from Thromboelastography (TEG)

	InTEM				ExTEM				FibTEM
compd reference range	*R* (s)[c] 100−240	*K* (s)[d] 30−110	∝ (deg)[e] 70−83	MA (mm)[f] 50−72	*R* (s)[c] 38−79	*K* (s)[d] 34−156	∝ (deg)[e] 63−83	MA (mm)[f] 50−72	MA (mm)[f] 9−25
control	188 ± 21	82 ± 3	77 ± 3	57 ± 2	67 ± 3	117 ± 34	67 ± 1	54 ± 2	10 ± 1
7	267 ± 7*[g]	229 ± 9*[g]	57 ± 5*[g]	45 ± 2*[g]	49 ± 3	150 ± 2*[g]	68 ± 5	54 ± 1	8 ± 1
8	300 ± 12*[g]	214 ± 32*[g]	57 ± 6*[g]	40 ± 2*[g]	59 ± 1	116 ± 23	70 ± 2	59 ± 3*[g]	8 ± 1
10	560 ± 82*[g]	122 ± 2*[g]	67 ± 1*[g]	64 ± 1*[g]	105 ± 7*[g]	111 ± 9	69 ± 2	61 ± 1*[g]	8 ± 1
13	261 ± 7*[g]	176 ± 43*[g]	61 ± 6*[g]	48 ± 2*[g]	120 ± 26*[g]	257 ± 85*[g]	51 ± 11*[g]	46 ± 2*[g]	7 ± 1

[a] Data points represent the average ± standard error of the mean (SEM) of three independent experiments done in duplicate. [b] At higher concentrations, no clot formation was observed. [c] *R*: clotting time expressed in seconds (s). [d] *K*: clotting formation time expressed in seconds (s). [e] ∝ angle expressed in degrees (°). [f] MA: maximum amplitude expressed in mm. [g] * $P < 0.05$.

concentration tested (0.5×10^{-3} M), while the sulfated aglycone **12** prolonged 1.1 fold at 5×10^{-3} M.

Prolongation of PT (Figure 3) was observed for compounds **7**, **8**, **10**, and **13** in a dose-dependent manner. Nonetheless, this effect was observed for higher concentrations than in APTT. Compounds **7**, **8**, **10**, and **13** were also able to double the PT (Figure 3 and Table 1) and, with one exception (compound **8**), were also able to completely inhibit this clotting pathway. The sulfated aglycones **11** and **12** only increased the PT 1.1 and 1.2 times, respectively (Figure 3), at the highest concentration tested (0.5×10^{-3} and 5×10^{-3} M, respectively).

The TT was sensitive to the presence of compounds **10** and **13** that were able to completely block this clotting process at 5×10^{-3} M (Figure 3 and Table 1). Compound **7** increased TT 1.6-fold at the highest concentration tested (5×10^{-3} M). The clotting times for flavonoside **8** (5×10^{-3} M), sulfated aglycones **11** (0.5×10^{-3} M), and **12** (5×10^{-3} M) were not significantly different ($P > 0.05$) from control (Figure 3).

In summary, compounds **7**, **8**, and **10**−**13** prolonged APTT and PT, but only compounds **10** and **13** influenced the TT pathway.

A structure−activity relationship can be established concerning the anticoagulant action and the number of sulfate groups, i.e., the anticoagulant effects increased with the increase of the number of sulfates. Decasulfated compound **13** was more potent than nonasulfated compound **10**, and compound **10** more than hexasulfated compounds **7** and **8**; the disulfated compounds **11** and **12** were only slightly active in prolonging the clotting times. The weak anticoagulant effect showed by sulfated aglycones **11** and **12** when compared to the related sulfated flavonosides **7** and **8** also highlights the importance of the oligo-*O*-sulfated moiety in the anticoagulant activity. Flavonosides **7**, **8**, **10**, and **13** were more potent than the previously described sulfated flavonoids.[23,42] Additionally, when comparing different classes of flavonoids, namely flavones (compounds **7** and **11**) with flavanones (compounds **8** and **12**, respectively), comprising the same number of sulfates, it can be noted a slightly preference for flavone scaffolds, in the prolongation of the clotting times, namely APTT.

Among the three clotting times, the APTT was the most sensitive test to the presence of sulfated compounds. On the basis of these results, flavonosides **10** and **13** may be implicated in the conversion of fibrin to fibrinogen by thrombin while flavonosides **7** and **8** may be acting similarly to heparin (prolonging APTT more than PT).

To better characterize the anticoagulant profile, the effect on the whole blood of the most promising sulfated molecules (**7**, **8**, **10**, and **13**) was studied by thromboelastography (TEG) (Table 2). In Figure 4, the TEG representative tracings for polysulfated flavonosides (e.g., compound **13**) are illustrated.

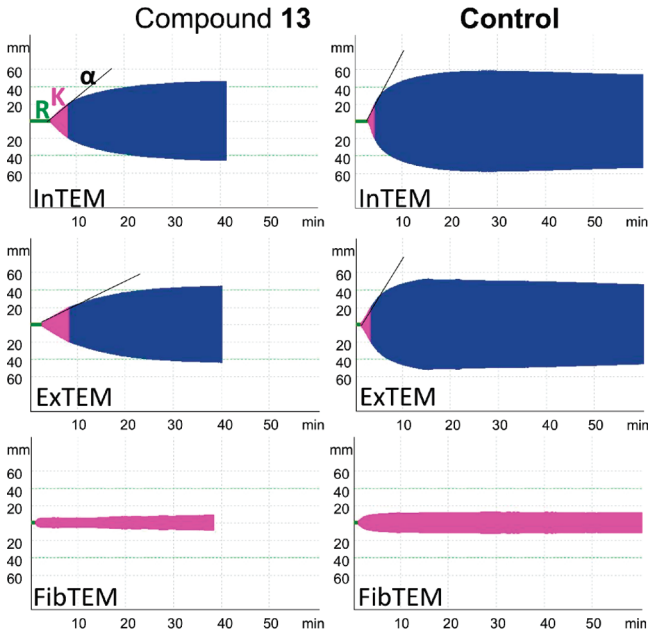


Figure 4. Thromboelastography (TEG) representative tracings of polysulfated flavonoside **13** (1.25×10^{-4} M).

While clotting tests evaluate one isolated segment of the coagulation system (secondary hemostasis), TEG provides data about the coagulation process as a whole, from the beginning of clot formation, through clot stabilization, and ending with fibrinolysis.[43] In contrast to plasma clotting tests, TEG screens the thrombodynamic properties of the entire blood. The clot is induced under a low shear environment resembling an in vivo sluggish venous flow.[43] Thus, TEG allows acquisition of quantitative information about the kinetics of clot formation and growth as well as the maximal strength and stability attained by clots (the quality of clots).[43] The parameters measured by TEG are: *R*, the period of time from initiation of the test to the initial fibrin formation (clotting time) and is due only to plasma factors; *K*, the time from beginning of clot formation until the amplitude of thromboelastogram reaches 20 mm (clotting formation time); ∝ angle, the acceleration/kinetics of fibrin build up and cross-linking (clot formation and growth); and MA, the strength and stability of the formed clot (maximum clot firmness/maximum amplitude strength of clot). Modified thromboelastogram was obtained by adding to the whole blood an intrinsic activator (InTEM) or an extrinsic activator (ExTEM). InTEM and ExTEM are global tests for plasmatic coagulation factors, fibrin polymerization, and platelet contribution. It was also possible to eliminate platelet function from the TEG

sample and study the fibrin status by adding cytochalasin D and Ca^{2+} (FibTEM).

In the presence of compounds **7**, **8**, **10**, and **13**, the thromboelastogram traces showed a hypocoagulable blood and there was no evidence of fibrinolytic activity (Figure 4). Compared to control values, compounds **7**, **8**, **10**, and **13** increase not only the clotting time (*R*) but also increase the clotting formation time (*K*) and decrease ∝ (deceleration progress of the clot) and MA values (Table 2). Because *K* and ∝ are a combination of both plasma and platelet factors and MA is largely dependent on platelet function and integrity of the fibrin clot, it is evident that an action on platelets may also be implicated in the antithrombotic effect of compounds **7**, **8**, **10**, and **13**. Moreover, normal thromboelastograms were obtained in the presence of these compounds when the platelet-specific effect on MA of TEG tracings is eliminated (FibTEM test).

From these data, it can be inferred that polysulfated flavonosides **7**, **8**, **10**, and **13** may interfere with coagulation cascade factors and/or platelet function. Therefore, their influence on platelets, thrombin, FXa, and ATIII were studied.

To measure platelet adhesion and aggregation in whole blood, a platelet function analyzer (PFA) was used.[44,45] In the PFA-100 system, the closure of a defined aperture is caused by platelets which are activated by a collagen-coated membrane and an additional agonist, adenosine 5′-diphosphate (ADP) or epinephrine (EPI). At the aperture exists a shear stress of about 5000/s, which is about twice the level inside a moderately stenosed artery. Platelets adhere at the aperture and form aggregates consisting of platelets bound to each other mainly by von Willebrand factor. When a clot is formed, further blood flow is prevented and this time is registered as the closure time. Hence, the closure time refers to the process of platelet plug formation, including adhesion and aggregation at high shear stress forces. The PFA-100 closure times in the presence of polysulfated flavonosides **7**, **8**, **10**, and **13** are collected in Table 3.

Table 3. Effect[a] of Polysulfated Flavonosides **7**, **8**, **10** (6.25×10^{-4} M), and **13** (1.25×10^{-4} M)[b] on Collagen-ADP or EPI Induced Platelet Aggregation in Human Whole Blood

compd reference range	collagen-ADP 71−118	collagen-EPI 94−193
control	119 ± 8	168 ± 5
7	162 ± 16*[c]	171 ± 3
8	139 ± 7*[c]	167 ± 17
10	204 ± 44*[c]	>300
13	189 ± 13*[c]	244 ± 45*[c]

[a] Results expressed in closure time expressed in seconds (s) and represent the average ± SEM of three independent experiments. [b] At higher concentrations, no clot formation was observed. [c] *$P < 0.05$.

Compounds **7** and **8** prolonged the collagen-ADP closure times. However, it is unlikely that a platelet dysfunction exists in the presence of compounds **7** and **8** because the collagen-EPI times were not significantly different ($P > 0.05$) from control. PFA-100 closure times of both collagen−ADP and collagen−EPI cartridges were markedly longer in the presence of compounds **10** and **13** (Table 3). Because results of both tests were abnormal, it is likely that compounds **10** and **13** caused a platelet dysfunction. Further studies should be performed to support the antiplatelet action of compounds **10** and **13**. The possibility of a dual anticoagulant and platelet antiaggregatory activities for compounds **10** and **13** could enhance their potential as antithrombotic agents.[46]

Compounds **7**, **8**, **10**, and **13** were further evaluated for selectivity against the critical enzymes targeted by current anticoagulant therapy, thrombin, FXa, and ATIII (Table 4). Inhibition of thrombin and FXa, in the presence and in absence of ATIII, was followed by spectrophotometric determination of the product formed after amide bond cleavage of the chromogenic substrates Chromozym TH and CBS 31.39, respectively and the initial rate compared with that obtained in the absence of the compound. Reference inhibitors, EDTA (direct FXa inhibitor), LMWH (ATIII/FXa activator), and

Table 4. In Vitro Inhibitory Activity[a] of Polysulfated Flavonosides **7**, **8**, **10**, and **13** against Human FXa and ATIII Inhibition of FXa in the Presence of CBS 31.39

	human FXa		ATIII/FXa	
substrate→	25×10^{-6} M	125×10^{-6} M	25×10^{-6} M	125×10^{-6} M
7				
2×10^{-3} M	nd	23.35 ± 2.91	43.69 ± 2.63	50.66 ± 2.64
1×10^{-3} M	na	na	22.58 ± 1.76	38.70 ± 5.48
0.5×10^{-3} M	na	na	na	28.91 ± 2.77
8				
2×10^{-3} M	na	na	49.56 ± 8.59	58.12 ± 2.91
1×10^{-3} M	na	na	40.18 ± 3.55	46.21 ± 5.68
0.5×10^{-3} M	na	na	30.50 ± 1.76	39.72 ± 3.38
10				
2×10^{-3} M	nd	53.27 ± 6.57	56.01	33.57 ± 4.87
1×10^{-3} M	41.94 ± 20.81	38.83 ± 7.81	47.65 ± 3.08	24.80 ± 1.67
0.5×10^{-3} M	25.66 ± 4.84	na	28.15 ± 1.06	20.30 ± 2.07
13				
2×10^{-3} M	40.18 ± 1.79	42.70 ± 3.49	31.38 ± 1.83	33.11 ± 1.22
1×10^{-3} M	30.94 ± 3.96	31.98 ± 0.94	30.50 ± 4.16	20.70 ± 0.92
0.5×10^{-3} M	na	22.4 ± 3.04	29.33 ± 1.17	na
EDTA				
10×10^{-3} M	45.74 ± 0.13	58.96 ± 6.98	73.51 ± 0.44	72.14 ± 1.38
1×10^{-3} M	na	na	nd	nd
LMWH				
0.20 U/mL	na	na	96.31 ± 0.51	97.96 ± 0.58

[a] Results expressed as percentage of inhibition; each value represents the mean ± SEM of three independent experiments. nd: not determined. na: not active at the concentration tested ($P > 0.05$).

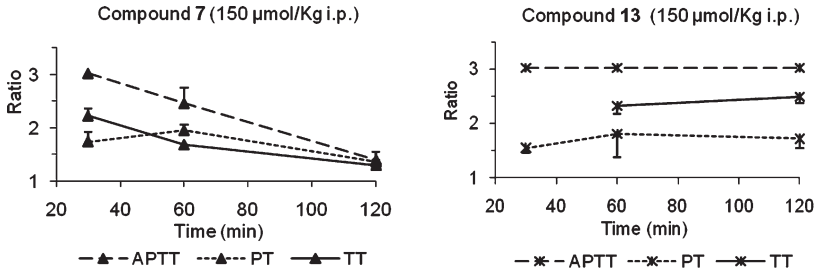


Figure 5. APTT, PT, and TT prolonging activities of compound **7** and **13** in mice at 150 μmol/kg, after 30, 60, and 120 min of ip administration, expressed as ratio of the clotting time of compound-treated mice to clotting time of saline-treated mice (control group). Data points represent the average ± SEM of three independent experiments.

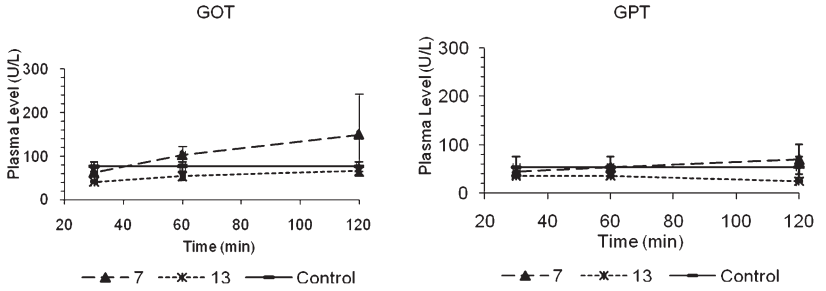


Figure 6. Plasma levels of glutamic-oxaloacetic transaminase (GOT) and glutamate-pyruvate-transaminase (GPT) in mice 30, 60, and 120 min after ip administration of compound **7** and **13** at 150 μmol/kg, expressed as U/L. Data points represent the average ± SEM of four independent experiments.

PPACK (direct thrombin inhibitor) were also tested in the same conditions. Various concentrations of the sulfated inhibitors (0.5, 1, and 2×10^{-3} M) and CBS 31.39 (25 and 125×10^{-6} M) were used (Table 4). Kinetic studies of the inhibitory activity of sulfated flavonosides **7**, **8**, **10**, and **13** against FXa and ATIII/FXa were fitted by the Michaelis−Menten equation (nonlinear regression analysis, see Supporting Information).

None of the compounds **7**, **8**, **10**, and **13** showed any influence on thrombin activity (% of PPACK thrombin inhibition at 1.9×10^{-9} M was equal to 37 ± 5%) even in the presence of ATIII (data not shown). Compounds **7**, **10**, and **13** showed a direct inhibitory effect on FXa (Table 4). For compound **7**, this effect was significantly ($P < 0.05$) enhanced by the presence of ATIII, suggesting an indirect action on FXa by activation of ATIII. Compound **8** showed an inhibitory effect on FXa only in the presence of ATIII. For compound **10** and **13**, the percentage of FXa inhibition in the presence of ATIII was not significantly different from blank ($P > 0.05$). Therefore, compounds **10** and **13** are direct FXa inhibitors. As previously discussed, compounds **7** and **8** may act similarly to heparin. The position of the oligo-*O*-sulfated moiety on the molecular scaffold seems to be correlated with this effect: while 3-*O*-rutinosides (compounds **10** and **13**) were direct FXa inhibitors, 7-*O*-rutinosides (compounds **7** and **8**) were ATIII activators of FXa inhibition. In the presence of compounds **10** and **13**, the V_{max} values did not change significantly, while the effective K_m increased, suggesting a competitive inhibition (see Supporting Information).

A high-priority goal in the search for new antithrombotic agents is the discovery of small molecules that selectively inhibit FXa or thrombin. Direct inhibitions of these targets have been suggested to be a better strategy because they do not require an intermediary protein to mediate the anticoagulant effect.[47] Three new oral drugs, rivaroxaban[48] and apixaban[49] as FXa direct inhibitors, and dabigatran etexilate,[50] as a thrombin direct inhibitor, were successfully developed.[51] Although direct thrombin inhibitors are able to inhibit clot-bound thrombin,[52] they are unable to block the continuing production of thrombin from prothrombin.[53,54] In contrast, FXa inhibitors prevent the conversion of prothrombin to thrombin and have no influence in the previously formed thrombin; they allow a basal level of thrombin activity necessary for primary hemostasis maintenance, resulting in less bleeding complications.[53,54] FXa inhibitors have advantages over thrombin inhibitors.[53,54] On the basis of these considerations, direct FXa inhibitors **10** and **13** are potentially safer alternatives to the existing anticoagulant agents.

Previously to in vivo assays in animals, stability studies in plasma were performed for one representative direct (**13**) and indirect (**7**) inhibitor of FXa. Three hours time point was selected to cover the time period used in the present in vivo and in vitro experiments. Compound **7** and **13** were stable after 3 h in human plasma (concentrations of at least 95% of the initial one were detected). This is consistent with the previously reported stability for rutin decasulfate (**13**).[55]

Subsequently, to assess the putative in vivo efficacy, we performed in vivo studies in mice to measure anticoagulant activity and putative hepatic toxicity for compounds **7** and **13**. To estimate the in vivo anticoagulant activity, APTT, PT, and TT were determined at 30, 60, and 120 min after 150 μmol/kg intraperitoneal (ip) administration or at 120 min after 300 μmol/kg oral administration by gavage. These values were compared with the APTT, PT, and TT values obtained with saline-treated mice groups and expressed as a ratio (Figure 5). Considering the previous reported hepatotoxic effects of heparin and LMWH,[9,56,57] it was of interest to estimate any possible drug-induced hepatocellular injury of these compounds. Plasma levels of glutamic-oxaloacetic transaminase/aspartate aminotransferase (GOT/AST) and glutamate-pyruvate-transaminase/alanine aminotransferase (GPT/ALT) were evaluated following the ip administration (Figure 6).[58]

After ip administration, both compounds **7** and **13** were highly active, increasing the APTT to the desirable therapeutic range (1.5−2.3 times the mean of the normal APTT range,[59] Figure 5). In contrast, heparin does not produce systemic anticoagulant action after ip administration.[60] Moreover, both compounds were still active after 120 min, particularly compound **13**, being reasonable to speculate that they will have a desirable sustained duration of action.[61,62] Because compounds **7** and **13** showed stability in plasma, the anticoagulant activity at 120 min cannot be attributed to decomposition products such as desulfonated byproduct or parent compounds. Furthermore, rutin decasulfate (**13**) has previously shown favorable pharmacokinetic profile.[55] After oral administration, none of the compounds tested (**7** and **13**) were active (results not shown). These results might be related to the previously reported behavior of rutin decasulfate (**13**) in Caco-2 cells.[63] If required, formulations could be developed to achieve oral administration because delivery agents have already demonstrated to improve oral absorption of macromolecules like heparin.[64] The rapid onset of action revealed by compounds **7** and **13** is a desirable feature compared to oral anticoagulants drugs, acenocoumarol and warfarin sodium.

Considering the plasma levels of transaminases, no significant alteration ($P > 0.05$) of GOT and GPT plasma levels was observed after administration of both compounds **7** and **13** (Figure 6). In general, after severe damage, GOT levels rise 10 to 20 times or higher than normal whereas GPT can reach levels up to 50 times greater than normal.[58] Thus, these preliminary results suggested that these compounds will not induce hepatic toxicity, in contrast to unfractionated and LMW heparins.[9,56,57]

From the overall results, these derivatives are expected to possess minimal toxicity and appear to have the potential to lead to effective and safe therapeutics.

Conclusion

The incorporation of an oligo-*O*-sulfated moiety into a flavonoid scaffold was a successful strategy to improve the anticoagulant potency of sulfated flavonoids. Polysulfated flavonosides proved to be active in vitro both in plasma and in whole human blood as well as in vivo, surpassing existing anticoagulant flavonoids. This study provided interesting SAR by revealing sulfated 7-*O*-rutinosides as indirect FXa inhibitors and sulfated 3-*O*-rutinosides as direct FXa inhibitors. In contrast to unfractionated and LMW heparins, the polysulfated oligoflavonoids correspond to compounds with a defined composition and with less charge density that allowed a systemic anticoagulant action after ip administration and are not expected to induce hemorrhagic complications and hepatic toxicity, as it was preliminarily proved with the in vivo studies in mice.

The derivatives resulting from chemical molecular modifications of known therapeutic agents have high chances to produce safer anticoagulant agents and this study provided interesting data to be explored by rational design in the future.

Experimental Section

Chemistry. Triethylamine−sulfur trioxide adduct (S 5139) was purchased from Fluka (Spain), and diosmin (**1**, D 3525), hesperidin (**2**, S 5139), rutin (**3**, R 2303), and trihydroxyethylrutin (**4**, 91950) were purchased from Sigma-Aldrich (Spain). The solvents used were products pro analysis or HPLC grade from Sigma-Aldrich and Fluka. Spectra/Por Dialysis membrane, MWCO 1000, was purchased from Spectrum Laboratories, Inc. (California, US). MW reactions were performed using a MicroSYNTH 1600 from Millestone (ThermoUnicam, Portugal) synthesizer in sealed reaction vessels (30 mL). TLC was performed using Polygram cel 400 UV_{254} 0.1 mm (Macherey-Nagel, Germany) ($BuOH-CH_3COOH-H_2O$ 4:2:6 and 5:2:3) and Merck silica gel 60 (GF254) plates ($CHCl_3$:MeOH 9:1). Compounds were visually detected by absorbance at 254 and/or 365 nm. Melting points were obtained in a Köfler microscope and are uncorrected. IR spectra were measured on an ATI Mattson Genesis series FTIR (software: WinFirst v.2.10) spectrophotometer in KBr microplates (cm^{-1}). ^1H and ^{13}C NMR spectra were taken in DMSO-d_6 at room temperature, on Bruker Avance 300 and 500 instruments (300.13 or 500.13 MHz for ^1H and 75.47 or 125.77 MHz for ^{13}C). Chemical shifts are expressed in δ (ppm) values relative to tetramethylsilane (TMS) as an internal reference. Coupling constants are reported in hertz (Hz). ^{13}C NMR assignments were made by 2D HSQC and HMBC experiments (long-range C, H coupling constants were optimized to 7 and 1 Hz). MS spectra were recorded as FAB (Fast Atom Bombardment) or EI (Electronic Impact) mode on a VG Autospec Q spectrometer (m/z), and HRMS mass spectra were measured on a APEX III mass spectrometer, recorded as ESI (Electrospray) mode in Centro de Apoio Científico e Tecnolóxico á Investigation (CACTI, University of Vigo, Spain). The purity of each compound was determined by HPLC-DAD analysis. Except for compound **9**, all tested compounds, whether synthesized or purchased, possessed a purity of at least 95%.

HPLC Chromatographic Conditions. The HPLC analyses were carried out on a System SMI pump series II (Gloucester, UK) equipped with a Rheodyne 7125 injector fitted with a 20 μL loop, a TSP-UV6000LP detector, and a Chromquest for Windows NT integrator and using a C-18 Nucleosil column (5 μm, 250 mm × 4.6 mm I.D.), from Macherey-Nagel (Düren, Germany). Acetonitrile was of HPLC grade from Merck. HPLC ultrapure water was generated by a Milli-Q system (Millipore, Bedford, MA, USA). The mobile phases were degassed for 15 min in an ultrasonic bath before use. The mobile phase used was 10 mM of phosphate buffer with 25 mM of TBA (adjusted to pH 8 with TEA) and acetonitrile (55:45) at a constant flow rate of 1.0 mL/min.

General Procedure for the Hydrolysis of Flavonoids Glycosides (Procedure a). Flavonoid glycosides **1** (3.29 mmol) and **2** (4.91 mmol) were kept under reflux in 200 mL of a mixture of sulfuric acid−acetic acid−water (1:30:19) for 12 h (see Supporting Information for experimental details of each purification process).

General Procedure for Sulfation with Conventional Heating (Procedure b). To a solution of a flavonoid (**1**−**6**, 0.8−3 mmol) in DMA (15 mL), triethylamine−sulfur trioxide adduct (4−8 equiv/OH) was added and the suspension was heated at 65 °C for 24 h. The mixture was poured into acetone (150 mL) under basic conditions (a few mL of triethylamine) and left at 4 °C for 24 h. The crude oil formed was washed with acetone and ether and then dissolved in aqueous solution of 30% sodium acetate (5 mL). Generally, the suspension was added dropwise in ethanol to precipitate the sodium salt of the sulfated derivative (see Supporting Information for experimental details of each syntheses and purification process).

General Procedure for Microwave-Assisted Sulfation (Procedure c). To a solution of a flavonoid (**1**−**4**, 0.8 mmol) in DMA (10 mL), triethylamine−sulfur trioxide adduct (10 equiv/OH) was added. The reaction vessel was sealed, and the mixture was kept stirring and heated for 30 min at 100 °C under MW irradiation. The reaction mixture was ramped to 100 °C using the following power −temperature steps: (1) 200 W, room temperature−80 °C, during 1 min; (2) 200 W, 80−100 °C, for 29 min. After cooling, the mixture was poured into acetone (150 mL) under basic conditions (a few mL of triethylamine) and left at 4 °C for 24 h. The crude oil formed was washed with acetone and ether and then dissolved in aqueous solution of 30% sodium acetate (5 mL). The suspension was added dropwise in ethanol to precipitate the sodium salt of the sulfated derivative **7** and **13**. In the case of compounds **8** and **10**, the solid obtained was further purified from other salts (monitored

by IR) by dialysis using a Spectra/Por 6 regenerated cellulose MWCO 1000.

Diosmin 2″,2‴,3″,3‴,4″,4‴-*O*-Hexasulfate (7). Yellow solid, yield 91% (procedure b) and 86% (procedure c); mp 184−185 °C (ethanol). IR (KBr) *v*max: 3600−3400, 1653, 1616, 1508, 1261, 1047, 806. ^{1}H NMR (DMSO-d_6, 500.13 MHz) δ: 12.83 (1H, s, 5-OH), 8.79 (1H, s, 3′-O*H*), 7.99 (1H, d, J = 2.2 Hz, H-2′), 7.89 (1H, dd, J = 8.8 and 2.2 Hz, H-6′), 7.24 (1H, d, J = 8.8 Hz, H-5′), 6.77 (1H, d, J = 1.9 Hz, H-8), 6.56 (1H, s, H-3), 6.39 (1H, d, J = 1.9 Hz, H-6), 5.71 (1H, d, J = 2.8 Hz, H-1‴), 5.02 (1H, d, J = 11.0 Hz, H-1″), 4.59−4.55 (3H, m, H-4″, H-2″ and H-4‴), 4.43 (1H, t, J = 3.5 Hz, H-3‴), 4.35 (1H, d br, J = 7.9 Hz, H-2‴), 4.27−4.25 (1H, m, H-5′), 4.03 (2H, t, J = 9.8 Hz, H-3″), 3.84 (3H, s, 4′-OCH_3), 3.80−3.50 (1H, m, H-5‴), 3.52−3.40 (2H, under H_2O, H-6″), 1.23 (3H, d, J = 6.2 Hz, H-6‴). ^{13}C NMR (DMSO-d_6, 125.77 MHz) δ: 181.9 (C-4), 164.1 (C-2), 162.6 (C-7), 161.2 (C-5), 157.1 (C-9), 154.2 (C-4′), 142.7 (C-3′), 123.2 (C-6′), 122.0 (C-1′), 118.7 (C-2′), 113.0 (C-5′), 105.5 (C-10), 103.5 (C-3), 99.7 (C-6), 97.9 (C-1‴), 97.5 (C-1″), 95.1 (C-8), 75.8 (C-3″), 75.3 (C-5″), 74.3 (C-2″), 74.0 (C-4‴), 73.4 (C-4″), 73.1 (C-2‴), 71.1 (C-3‴), 68.8 (C-5‴), 67.4 (C-6″), 55.9 (4′-OCH_3), 18.0 (C-6‴). MS (FAB$^+$): m/z 1245 ([M + Na + H]$^+$). HRMS (ESI$^+$): m/z calcd for $C_{28}H_{26}O_{33}S_6Na_7$ 1242.79645, found 1242.80127.

Hesperidin 2″,2‴,3″,3‴,4″,4‴-*O*-Hexasulfate (8). Orange crystals, yield 70% (procedure b) and 58% (procedure c); mp 155−157 °C (water). IR (KBr) *v*max: 3600−3400, 1641, 1518, 1254, 1050, 805. ^{1}H NMR (DMSO-d_6, 300.13 MHz) δ: 11.99 (1H, s, 5-O*H*), 8.90 (1H, s, 3′-O*H*), 7.96 (1H, d, H-2′), 7.04−6.89 (2H, H-6′ and H-5′), 6.07 (2H, H-8 and H-6), 5.67−5.52 (1H, m, H-2), 4.93 (1H, brs, H-1‴), 4.43 (3H, s, 4′-OCH_3), 4.20 (1H, d, J = 9.0 Hz, H-1″), 4.05−3.71 (10H, rutinose), 3.10 (2H, dd, J = 14.5 and 7.3 Hz, H-3), 1.23 (3H, d, J = 6.1 Hz, H-6‴). ^{13}C NMR (DMSO-d_6, 75.47 MHz) δ: 197.1 (C-4), 164.7 (C-7), 163.1 (C-5), 162.8 (C-9), 148.0 (C-4′), 146.5 (C-3′), 131.2 (C-1′), 130.2 (C-6′), 117.9 (C-2′), 114.1 (C-5′), 112.2 (C-10), 103.5 (C-1‴), 96.4 (C-1″), 95.4 (C-6), 91.5 (C-8), 78.4 (C-2), 75.8 (C-3″), 74.2 (C-5″), 73.5 (C-2″), 72.3 (C-4‴), 71.5 (C-4″), 70.9 (C-2‴), 68.9 (C-3‴), 67.5 (C-5‴), 61.5 (C-6″), 55.7 (4′-OCH_3), 45.9 (C-3), 15.2 (C-6‴). MS (FAB$^+$): m/z 1244 ([M + Na + H]$^+$). HRMS (ESI$^+$): m/z calcd for $C_{28}H_{28}O_{33}S_6Na_7$ 1244.81155, found 1244.81504.

Rutin 2″,2‴,3′,3″,3‴,4′,4″,4‴,7-*O*-Nonasulfate (9). Yellow solid, yield 24%(procedure b, purity <95%); mp >340 °C (water). IR (KBr) *v*max: 1652, 1615, 1500, 1258, 1055, 808. ^{1}H NMR (DMSO-d_6, 500.13 MHz) δ: 12.60 (1H, s, 5-O*H*), 8.14 (1H, d, J = 8.9, H-2′), 8.06 (1H, m, H-6′) 7.65 (1H, d, J = 8.9, H-5′), 6.89 (1H, s, H-8), 6.53 (1H, s, H-6), 4.94 (1H, d, J = 10.2, H-1″), 4.91 (1H, s, H-1‴), 4.76−4.03 (10H, rutinose), 1.24 (3H, d, J = 5.2, H-6‴). ^{13}C NMR (DMSO-d_6, 125.77 MHz) δ: 172.0 (C-4), 160.2 (C-7), 159.1 (C-5), 156.9 (C9), 156.4 (C-2), 146.7 (C-4′), 143.3 (C-3′), 133.7 (C-3), 125.3 (C-6′), 122.6 (C-1′), 119.8 (C-5′), 118.1 (C-2′), 104.2 (C-10), 101.9 (C-1″), 100.3 (C-1″) 99.1 (C-6), 97.0 (C-8), 81.1−66.9 (9C-rutinose), 17.9 (C-6‴).

3″,4″-Bis(2-*O*-sulfate ethoxy)-7-(2-*O*-sulfate ethoxy)-rutin (10). Orange solid, yield 20% (procedure b) and 89% (procedure c); mp 193−195 °C (ethanol). IR (KBr) *v*max: 3600−3400, 1649, 1610, 1258, 1059, 800. ^{1}H NMR (DMSO-d_6, 300.13 MHz) δ: 12.62 (1H, s, 5-O*H*), 7.96 (1H, m, H-6′), 7.65 (1H, d, J = 2.0 Hz, H-2′), 7.24 (1H, d, J = 8.8 Hz, H-5′), 6.93 (1H, d, J = 2.0 Hz, H-8), 6.36 (1H, d, J = 2.0 Hz, H-6), 5.68 (1H, d, J = 3.3 Hz, H-1″), 4.41 (1H, s, H-1‴), 4.27 (6H, m, OCH_2CH$_2$OSO$_3^-$), 4.11 (6H, m, OCH$_2$-CH_2OSO$_3^-$), 3.99−3.07 (10H, rutinose), 0.98 (3H, d, H-6‴). ^{13}C NMR (DMSO-d_6, 75.47 MHz) δ: 177.6 (C-4), 164.2 (C-7), 160.7 (C-5), 156.6 (C-2), 156.4 (C-9), 150.5 (C-4′), 147.6 (C-3′), 134.4 (C-3), 124.2 (C-2′), 122.3 (C-6′), 120.0 (C-1′), 113.6 (C-2′), 113.0 (C-6′), 112.8 (C-5′), 105.3 (C-10), 100.7 (C-1‴), 100.5 (C-1″), 98.9 (C-6), 98.5 (C-8), 73.92−64.10 (9C, rutinose), 56.07 (6C-OCH_2CH_2OH), 45.80 (6C-OCH_2CH_2OH), 17.78 (C-6‴). HRMS (ESI$^+$): m/z calcd for $C_{33}H_{33}O_{46}S_9Na_{10}$ 1682.67, found 1682.67.

Diosmetin 3′,7-*O*-Disulfate (11). Yellow solid, yield 14% (procedure b); mp >340 °C (ethanol). IR (KBr) *v*max: 3600−3400, 2963, 2875, 1655, 1606, 1510, 1489, 1261, 1043. ^{1}H NMR (DMSO-d_6, 300.13 MHz) δ: 12.81 (1H, s, 5-O*H*), 8.09 (1H, d, J = 2.0 Hz, H-2′), 7.82 (1H, dd, J = 8.7 and 2.0 Hz, H-6′), 7.18 (1H, d, J = 8.7 Hz, H-5′), 7.01 (1H, d, J = 1.8 Hz, H-8), 6.75 (1H, s, H-3), 6.57 (1H, d, J = 1.8 Hz, H-6), 3.86 (3H, s, 4′-OCH_3). ^{13}C NMR (DMSO-d_6, 75.47 MHz) δ: 182.0 (C-4), 164.0 (C-7), 160.5 (C-2), 159.7 (C-5), 156.4 (C-9), 154.1 (C-4′), 142.8 (C-3′), 122.7 (C-6′), 122.1 (C-1′), 118.7 (C-2′), 112.7 (C-5′), 105.7 (C-10), 103.7 (C-3), 102.3 (C-6), 97.7 (C-8), 55.8 (OCH_3). MS (FAB$^+$): 426 ([M − OCH$_3$]). HRMS (ESI$^-$) m/z calcd for $C_{16}H_{10}O_{12}S_2$ 457.98123, found 457.98071.

Hesperetin 3′,7-*O*-Disulfate (12). Brown solid, yield 5% (procedure b); mp >340 °C (ethanol). IR (KBr) *v*max: 3600−3400, 1622, 1637, 1516, 1043, 1265, 798. ^{1}H NMR (DMSO-d_6, 500.13 MHz) δ: 12.51 (1H, s, 5-O*H*), 7.55 (1H, d, J = 2.0 Hz, H-2′), 7.17 (1H, dd, J = 8.6 and 2.0 Hz, H-6′), 7.0 (1H, d, J = 8.6 Hz, H-5′), 6.89 (1H, d, J = 2.2 Hz, H-8), 6.71 (1H, d, J = 2.2 Hz, H-6), 5.4 (1H, d, J = 12 Hz, H-2), 3.78 (3H, s, 4′-OCH_3), 2.90 (2H, m, under H_2O, H-3). MS (EI$^+$): 430 ([M-OCH$_3$]). HRMS (ESI$^-$): m/z calcd for $C_{16}H_{12}O_{12}S_2$ 462.97617, found 462.77777.

Rutin 2″,2‴,3′,3″,3‴,4′,4″,4‴,5, 7-*O*-Decasulfate (13). White solid, yield 99% (procedure c); mp 222−225 °C (ethanol). IR (KBr) *v*max: 1643, 1515, 1430, 1265, 1024, 820. ^{1}H NMR (DMSO-d_6, 300.13 MHz) δ: 8.28 (1H, dd, J = 2.2 and 8.9 Hz, H-6′), 8.18 (1H, d, J = 2.2 Hz, H-2′), 7.65 (1H, d, J = 8.9 Hz, H-5′), 7.26 (1H, d, J = 2.2 Hz, H-8), 7.11 (1H, d, J = 2.2 Hz, H-6), 5.88 (1H, d, J = 2.0 Hz, H-1″), 5.15 (1H, s, H-1‴), 4.85−3.88 (10H, rutinose), 1.23 (3H, d, J = 6.2 Hz, H-6‴). ^{13}C NMR (DMSO-d_6, 75.47 MHz) δ: 172.9 (C-4), 157.5 (C-7), 156.2 (C-5), 152.6 (C-9), 152.4 (C-2), 146.3 (C-4′), 143.6 (C-3′), 137.6 (C-3), 125.5 (C-6′), 123.8 (C-1′), 120.2 (C-5′), 119.1 (C-2′), 110.8 (C-10), 107.9 (C-1″), 101.8 (C-1‴) 99.7 (C-6), 97.6 (C-8), 76.9−66.9 (9C, rutinose), 18.0 (C-6‴). HRMS (ESI$^+$): m/z calcd for $C_{27}H_{20}O_{46}S_{10}Na_{11}$ 1653.9576, found 1652.53247.

Biological Activity. Clotting Assays. Human blood was collected from 10 healthy donors aged between 25 and 45 years old without history of bleeding or thrombosis and who had not taken any medication known to affect blood coagulation and platelet function for 2 weeks. Venous blood was obtained and transferred to a plastic tube. Nine volumes of blood were decalcified with one volume of 3.8% sodium citrate solution. Blood was centrifuged for 20 min at 2400*g*, and the pooled plasma was stored at −20 °C until use. Compound **9** was not obtained with the desirable purity to be tested in biological assays. Sulfated compounds **7**, **8**, and **10**−**13** were dissolved in water. The final concentration of sulfated compounds in these assays ranged from 5×10^{-3} M to 0.05×10^{-3} M. Sulfated derivative **11** was not tested at 5×10^{-3} M because it was not soluble in water at 1×10^{-2} M. Compounds **2**−**4** were tested at a single concentration (5×10^{-3} M, 10% DMSO). Heparin (0.05−5 UI/mL) was used as positive control. In the control group, water (control for sulfated compounds **7**, **8**, and **10**−**13**) or 10% DMSO (control for desulfated compounds **2**−**4**) was used. APTT, PT, and TT tests were performed using an ACL100 coagulometer (IZASA, Portugal). The following commercial kits were used: 49735320 (IZASA, Portugal) for the APTT, 20002900 (IZASA, Portugal) for the PT, and 9758515 (IZASA, Portugal) for the TT. The assays were carried out according to the respective instructions of the manufacturers. For APTT assay, citrated normal human plasma (25 μL) was mixed with 25 μL of sample solution at various concentrations and incubated for 2 min at 37 °C. Then APTT assay reagent (56 μL) was added and the mixture was incubated for 6 min at 37 °C. $CaCl_2$ (50 μL, 25 mmol/L) was added, and clotting times were recorded during 110 s. The PT assay was carried out as follows: citrated normal human plasma (25 μL) was mixed with 25 μL of a solution of sample at various concentrations and incubated for 2 min at 37 °C. Then PT assay reagent (100 μL), preincubated for 2 min at 37 °C, was added and clotting times were recorded during

165 s. For TT assay, citrated normal human plasma (25 μL) was mixed with 25 μL of sample solution at various concentrations and incubated for 2 min at 37 °C. Then 100 μL of thrombin solution (100 U/mL), preincubated for 5 min at 37 °C, was added and clotting times were recorded during 165 s. The clotting times were recorded in seconds. Each measurement was performed in duplicate and repeated three times on different days ($n = 6$). Coagulation time prolonging ratio was calculated comparing the clotting time in the presence of each tested compound with that obtained when water was used instead of test compound. The concentration required to double the clotting time was calculated from linear regression analysis of each individual concentration−response curve.

Thromboelastography. Blood samples were collected from healthy donors into siliconized Vacutainer tubes containing 3.8% trisodium citrate such that a ratio of citrate/whole blood of 1:9 (v/v) was maintained and TEG was performed within 5 h of blood collection. The TEG assay was performed according to the manufacturer's guidelines using a thromboelastography coagulation analyzer Rotem delta (Pentapharm GmbH, Germany). All disposables supplies were purchased from Pentapharm GmbH. Sulfated compounds were dissolved in saline and diluted in the whole blood (1:16) to a final concentration of 6.25×10^{-4} M (compounds **7**, **8**, and **10**) or 1.25×10^{-4} M (compound **13**). In the control group, saline was added to the whole blood (1:16). As soon as each compound was added to the blood, the program was activated and allowed to run until 60 min. Clot formation is monitored at 37 °C in an oscillating plastic cylindrical cuvette (cup) and a coaxially suspended stationary piston (pin). During clot formation, fibrin fibrils physically link the cup to the pin and the pin starts to move. The movement of the pin is recorded as amplitude (mm). The stronger the clot, the more the pin moves with the cup and the higher MA is. InTEM and ExTEM were performed by adding a contact activator or tissue factor, respectively, and FibTEM, by adding cytochalasin D and Ca^{2+}.

Platelet Function. In vitro platelet function was evaluated using a PFA-100 device (Dade Behring, Frankfurt, Germany) according to the manufacturer's instructions. Compound solution (125 μL) was added to whole blood (1875 μL) to a final concentration of 6.25×10^{-4} M (compounds **7**, **8**, and **10**) or 1.25×10^{-4} M (compound **13**). This mixture was divided and applied into two cartridges. Both cartridges hold a membrane with a central aperture and are coated with collagen; one cartridge contains epinephrine and the other ADP. The time required for platelets to occlude the central aperture in the membrane is reported as the closure time.

Chromogenic Substrate Hydrolysis Assays. Human FXa (27 nkat/mL) and human thrombin (137 NIH/mL) were obtained from Diagnostica Stago (Roche, Portugal). Bovine ATIII was obtained from Sigma-Aldrich (Germany) (50 UI/mL). The chromogenic substrates used were Chromozym TH (Tosyl-Gly-Pro-Arg-*p*-nitroaniline(*p*NA), Roche Applied Science) for thrombin and CBS 31.39 (CH_3SO_2-D-Leu-Gly-Arg-*p*NA, AcOH; Diagnostica Stago) for FXa. Stock solutions of the enzymes were obtained by restitution according to the manufacture instructions and assay solutions were prepared by fresh dilution with the assay buffer. The assay buffer was 50 mM Tris/HCl at pH 8.3, containing 227 mM NaCl and 0.1% (w/v) bovine serum albumin (BSA, Sigma-Aldrich) instead of PEG-8000. The final enzyme concentrations were 0.5 U/mL, 0.06 U/mL, and 1 U/mL for FXa, thrombin, and ATIII, respectively. The final substrate concentrations in the reactions were 178×10^{-6} M and 125×10^{-6} M (in water) for Chromozym TH and CBS31.39, respectively. For the kinetic studies of CBS 31.39 hydrolysis by FXa in the presence of compounds **7**, **8**, **10**, and **13**, various concentrations of the sulfated inhibitors (0.5, 1, and 2×10^{-3} M) and CBS 31.39 (25 and 125×10^{-6} M) were investigated. Under the experimental conditions, less than 10% of the substrate was consumed in all assays [$\varepsilon_{405\ nm}$ (*p*-nitroaniline) = 10.4 $mmol^{-1} \cdot L \cdot cm^{-1}$]. The chromogenic assays were carried out at 37 °C in 96-well plates on a microplate reader (BioTeck Instruments, Vermont, US). Test compounds were diluted in water. Reference inhibitors PPACK (direct thrombin inhibitor, D-Phe-L-Pro-L-Arg-CMK), LMWH (ATIII/FXa activator), and EDTA (direct FXa inhibitor) were assayed in the same conditions. A 40 μL of test compound at 1 mM, final was diluted with 70 μL of buffer and warmed at 37 °C for 2 min before the addition of 20 μL of ATIII solution. For the direct inhibition of test compounds on thrombin or FXa, buffer was incorporated in place of ATIII solution. After the mixture was incubated at 37 °C for 10 min, 20 μL of thrombin solution or FXa solution was added. After 10 min of incubation at 37 °C, the enzyme reactions were initiated by the addition of chromogenic substrate, immediately placed in the microplate reader and the color developed from the release of *p*-nitroaniline from each substrate was monitored every 12 s for 20 min at 405 nm on a microplate reader. Absorbance was calculated by subtracting the absorbance at each point with the absorbance measured without the enzyme. The control was performed using water in place of the test compound. The initial slope of the absorbance curve in the presence of the test compound was compared to that of the control to obtain the percentage of inhibition. Data were fitted by the Michaelis−Menten equation. Measurements were carried out in duplicate and repeated at least three times on different days ($n = 6$).

Stability Studies. The plasma stability of tested compounds, at 25 μM final concentration, was performed in human plasma previously diluted 1:1 with phosphate buffer (PBS, pH 7.4). Three independent samples, plus respective blank and controls, were analyzed for each time (time zero and 180 min). Incubations were performed in eppendorfs, on a bath shaker, at 37 °C for 180 min. After the addition of tetrabutylammonium (TBA) buffer (10 mM potassium dihydrogen phosphate and 25 mM TBA, adjusted to pH 8 with TEA), the reaction was quenched with 4× cold acetonitrile (HPLC grade), followed immediately by mixing and centrifugation during 15 min at 14000 rpm. The time zero samples were quenched immediately after the sample was added to plasma. After filtration (Millipore), the supernatant was evaporated and the residue dissolved with mobile-phase (50 μM final concentration), and transferred to HPLC vials.

In Vivo Studies. Animals were kept in polyethylene cages with wood shavings as bedding and maintained in a temperature-controlled room at 20 ± 1 °C, with a 12/12 h lighting schedule (lights on at 08:00 h, off at 20:00 h), and relative humidity of 50% for at least 2 weeks prior to use. Water was available ad libitum at all times, but food was not available at the day of the experiments. Housing and experimental treatment of the mice were conducted under the European Community guidelines for the use of experimental animals (European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes, 1986, and Protocol of Amendment to the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes, 1998). All experiments were performed using adult male CD1 mice (30−35 g body wt; Charles River Laboratories, Criffa, Barcelona, Spain). Animals were grouped and housed, four per cage/group. The tested drugs were dissolved in saline (0.9% NaCl) and administered to animals ip at 150 μmol/kg or orally at 300 μmol/kg, by gavage, in a volume of 0.1 mL/40 g body weight. Citrated blood was collected from the portal vein into syringes containing citrate, 30, 60, and 120 min after ip administration, or 120 min after oral administration. Platelet-poor plasma was prepared by centrifugation for measurement of APTT, PT, and TT and transaminases levels (GOT, GPT). Levels of GOT and GPT were measured on the Roche Cobas Mira Plus automated chemistry analyzer. All data were expressed as relative fold values, compared with the values obtained with control group.

Data were tested for statistical significance by nonparametric two-tailed Mann−Whitney test using GraphPad Prism 5 software. A value of $P < 0.05$ was considered significant.

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Supporting Information Available: Structural characterization, experimental details in synthesis, purification, and Michaelis−Menten fit of in vitro enzyme inhibition assays. This material is available free of charge via the Internet at http://pubs.acs.org.

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