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Polysulfated Xanthones: Multipathway Development of a New Generation of Dual Anticoagulant/Antiplatelet Agents

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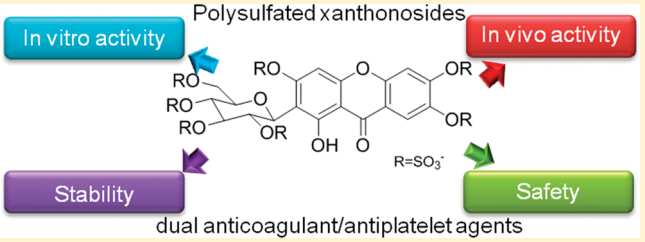
Supporting Information

ABSTRACT: A multipathway strategy was used to evaluate the in vitro and in vivo antithrombotic effects of a new synthetic family of sulfated small molecules. Polysulfated xanthonosides showed highly effective anticoagulation effects in vitro, both in plasma (clotting times) and in whole human blood (thromboelastography), as well as in vivo (ip administration, mice). Physicochemical properties were assessed for mangiferin heptasulfate (**7**), which showed high solubility and stability in water and in human plasma and no putative hepatotoxicity in vivo. Mangiferin heptasulfate (**7**) was found to be a direct inhibitor of FXa, while persulfated 3,6-(*O*-β-glucopyranosyl)xanthone (**13**) acted as a dual inhibitor of FXa (directly and by antithrombin III activation). By impedance aggregometry, compounds **7** and **13** exhibited the antiplatelet effect by inhibition of both arachidonic acid and ADP-induced platelet aggregation. Dual anticoagulant/antiplatelet agents, such as sulfated xanthonosides **7** and **13**, are expected to lead to a new therapeutic approach for the treatment of both venous and arterial thrombosis.



INTRODUCTION

Past research has highlighted the drawbacks of the therapeutic use of heparins.[1−4] Their anionic nature can result in significant interactions with multiple physiologically important proteins, leading to many side effects.[5,6] Heparin suffers from low tissue permeability, short serum half-life, and poor stability.[1] Consequently, the pharmacokinetic properties of heparin make it only adequate for intravenous application.[7] Commercially available heparin is a natural product obtained from either bovine or porcin sources, adding concerns about its structure variability[8] and the risk of contamination with animal pathogens.[9] The identification of a small sequence in the molecule of heparin, which is responsible for the affinity of heparin for antithrombin III (ATIII), has led to massive work in the past 2 decades devoted to the synthesis of oligosaccharides, especially DEFGH and its derivatives.[10−12] While some synthetic heparin-mimetic oligosaccharides show activity comparable to that of heparin, these molecules require a large number of synthetic steps, making them costly and possibly preventing their widespread clinical use.[10−12] Other sulfated saccharides, while simpler to synthesize, require a high level of charge to show a significant anticoagulant effect.[10−12]

On the basis of these considerations, our research has been focused on developing new small carbohydrate-based molecules that could preserve some molecular properties of the polysaccharide anticoagulant agents but with reduced anionic character, higher hydrophobic nature, and feasible synthesis. In this direction, we have recently obtained a series of polysulfated flavonosides (Figure 1) that combines both oligosulfated and flavonic moieties.[13] These compounds exhibited in vitro and in vivo anticoagulant activity, and preliminary toxicological studies suggested lack of acute side effects.[13]

Following these stimulating results, with the same purpose, we decided to obtain a new group of synthetic small molecules. A multipathway strategy of simultaneous investigation of parameters like stability, toxicity, and in vivo efficacy was followed to associate appropriate physicochemical properties to the structures of the newly synthesized compounds as early as possible in the discovery process.

Because of their similarity, we reasoned that sulfated xanthonosides may exhibit similar activities to sulfated flavonosides

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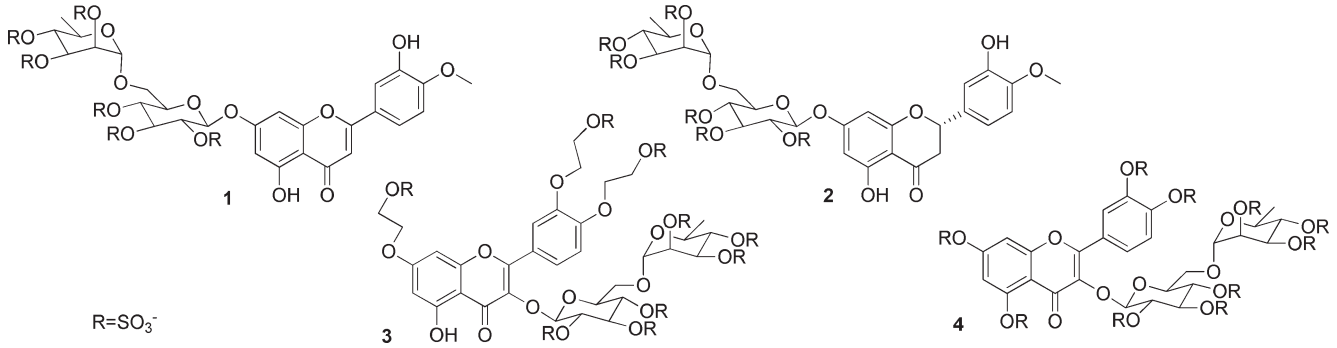

Figure 1. Polysulfated flavonosides with anticoagulant activity.[13]

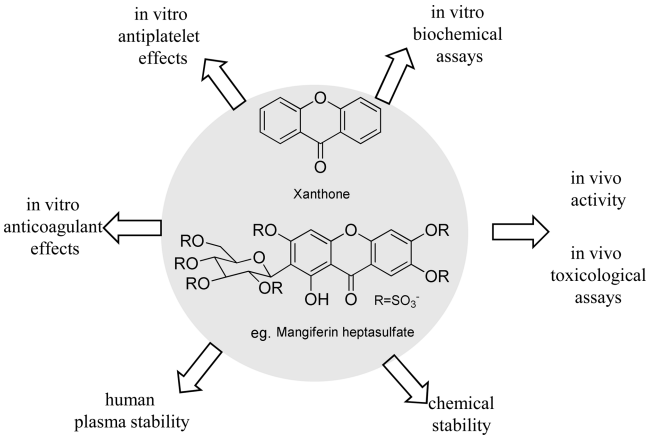

Figure 2. Schematic development of a new generation of cardiovascular agents of xanthonic nature.

(Figure 2).[14] Xanthones have a dibenzo-γ-pyrone nucleus and include a large group of compounds, both of natural[15] and synthetic origin[16] with a wide range of biological and pharmacological activities.[17] These derivatives have been reviewed for their cardiovascular effects[18] and described as antiplatelet,[19−21] antiarrhythmic, hypotensive,[22] and antithrombotic agents.[23,24] Nonetheless, few reports can be found concerning sulfated derivatives of xanthones.[25−27]

Mangiferin (**5**, Scheme 1) was selected for sulfation as part of our top priority of developing new molecules derived from molecular modifications of known drugs,[28] for several reasons that follow. Modification of molecules that have already been used in human therapy leads to compounds with more predictable and less complex pharmacokinetics, lower incidence of side effects, and less demanding clinical studies.[29] Mangiferin is a natural product and has been reported to be endowed with several pharmacological activities including antioxidant,[30] antiviral, prevention of carcinogenesis,[31] and inhibition of platetet aggregation.[21] Because of several interesting effects, mangiferin has been widely used as a nutritional supplement in cosmetic products and natural medicine.[32] The wide use of mangiferin by humans for many years suggests low toxicity not only for mangiferin but also for their metabolites and analogues. Protective effects of mangiferin with regard to cardio,[33,34] renal,[33] liver, and brain[35] toxicity are well documented. Furthermore, mangiferin is a *C*-glycoside. Because of favorable pharmacological profiles attributed primarily to the *C*-glycosyl moiety, aryl *C*-glycosides have gained increasing popularity as drug candidates.[36] Because aryl *C*-glycosides are stable analogues of *O*-glycosides,[36] resistant to metabolic processes, the *C*-glycoside mangiferin emerged as an interesting derivative to be sulfated.

A xanthone *O*-glycosylated on both rings (**12**, Scheme 1) was also planned to interact in an extended binding area of proteins that are critical in the coagulation process, such as ATIII.[37] According to this hypothesis, the sulfated diglycosylxanthone (**13**, Scheme 1) would lead to a molecule with higher molecular size and number of sulfate groups than the previous polysulfated flavonosides and thus increase anticoagulant effects.

In this work, the development of new sulfated xanthones having anticoagulant and antiplatelet properties is described. Compounds were screened by in vitro clotting time assays in human plasma and their effects in all components of human whole blood evaluated with thromboelastography. In order to understand the mechanism of action of the synthesized compounds, enzyme inhibition assays were performed against factor Xa (FXa), thrombin, prothrombin, activated protein C, and ATIII. The platelet aggregation was also investigated in whole blood with the platelet function analyzer (PFA-100) and with the new impedance aggregometer Multiplate. In vivo studies in mice were conducted for the direct FXa inhibitor to determine in vivo anticoagulant activity and toxicity potential.

CHEMISTRY

The synthesis of sulfated xanthones was first described in 2006 by a biotransformation of 1-hydroxy-2,3,5-trimethoxyxanthone to 5-sulfate-1-hydroxy-2,3-dimethoxyxanthone and 5-sulfate-1-hydroxy-2,3,7-trimethoxyxanthone in the *Trichothecium roseum* fungi.[26] A chemical procedure using pyridine−sulfur trioxide adduct was first described by some of us in the synthesis of 3,6-disulfate xanthone (**3**, Scheme 1).[27] Herein, three sulfated xanthones (**6**, **7**, and **13**) and one diglycosylxanthone (**12**) were obtained for the first time, in moderate yields (Scheme 1).

Sulfation of commercially available mangiferin (**5**) with 4 equiv/OH of triethylamine−sulfur trioxide adduct in dimethylacetamide at 65 °C (Scheme 1, step a) afforded mangiferin 2′,3′,4′,6′-tetrasulfate (**6**), and the use of higher quantities of sulfating agent (Scheme 1, step b) furnished the 2′,3,3′,4′,6,6′,7-heptasulfated derivative **7**. In both products (**6** and **7**), sulfation did not take place at the 5-hydroxyl group, as this is particularly nonreactive because of the formation of a strong hydrogen bond with the adjacent carbonyl at the 4-position.

To obtain the sulfated derivative **13**, the building block **12** was initially synthesized (Scheme 1, steps c−f). Despite the wide occurrence and biological importance of flavonoids and other

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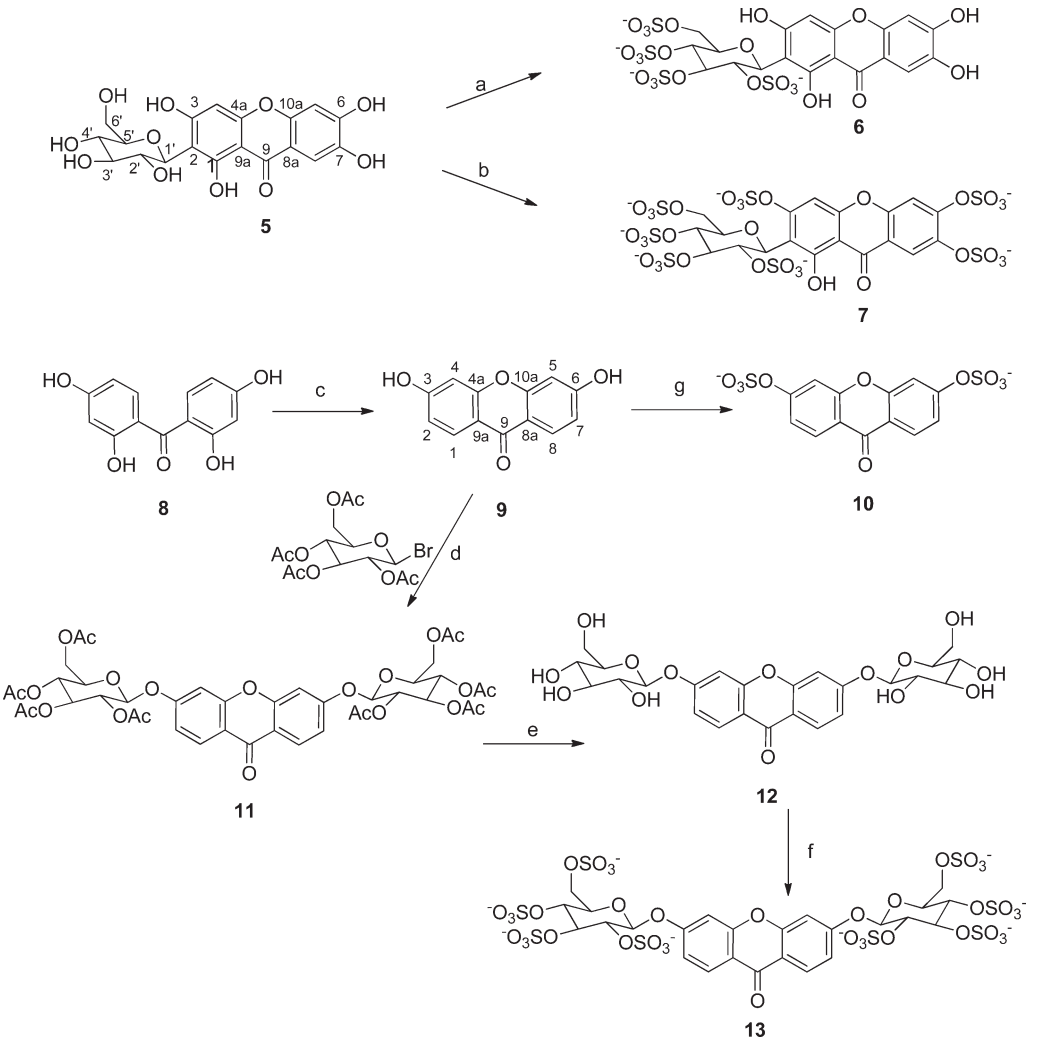
Scheme 1. Synthesis of Sulfated Glycosylxanthones 6, 7, and 13[a]



[a] Reagents and conditions: (a) SO_3/Et_3N (4 equiv/OH), DMA, 65 °C, 24 h; (b) SO_3/Et_3N (8 equiv/OH), DMA, 65 °C, 24 h; (c) furnace, 220 °C, overnight; (d) 0.15 M aq K_2CO_3, TBHAS (2 equiv/OH), $CHCl_3$, 50 °C, 36 h; (e) 0.08 M NaOMe, MeOH, room temp, overnight; (f) SO_3/Et_3N (6 equiv/OH), MW, 30 min; (g) Py/SO_3, DMA, 65 °C.[27] The numbering used corresponds to the NMR assignments.

polyphenol glycosides, synthetic efforts toward efficient preparation of this group of natural products are rarely reported. 3,6-Dihydroxyxanthone (**9**) was first obtained from 2,2′,4,4′-tetrahydroxybenzophenone (**8**) by a dehydrative process in the furnace (220 °C) (Scheme 1, step c).[38] Following, 3,6-(*O*-*β*-glucopyranosyl)xanthone (**12**) was obtained by phase transfer catalyzed (PTC) coupling reaction of 3,6-dihydroxyxanthone (**9**) and acetobromo-*β*-D-glucose in aqueous K_2CO_3 and TBAHS at 50 °C (Scheme 1, step d).[39] The same reaction was first conducted in dry DMF with anhydrous K_2CO_3 at room temperature. However, the product was not isolated, probably because of the easy hydrolysis of the acetyl groups from xanthone under these coupling conditions. 3,6-(*O*-*β*-Glucopyranosyl)xanthone (**12**) was obtained after deprotection of the glycosylated xanthone 3,6-(2,3,4,6-tetra-*O*-acetyl-*β*-D-glucopyranosyl)xanthone (**11**) with sodium methoxide in an overall 50% yield (Scheme 1, step e). Sulfation of compound **12** using microwave (MW) irradiation (Scheme 1, step f) allowed us to obtain the persulfated 3,6-(*O*-*β*-glucopyranosyl)xanthone (**13**) in 30 min and with 60% yield. The structure elucidation of compounds **6**, **7**, **12**, and **13** was established on the basis of IR, HRMS, and NMR techniques (see Supporting Information for detailed discussion).

The potential of druglikeness of the synthesized polysulfated glucosidic molecules was investigated by determining parameters, namely, solubility, chemical stability, and plasma stability which are essential for preformulation. Stability of polysulfated small, aromatic molecules has been rarely studied. The stability of resveratrol 4′-sulfate was investigated for 24 h at 37 °C in RPMI 1640 cell culture medium which was used for MCF-7 cellular uptake studies. While resveratrol 4′-sulfate was stable for 24 h, the nonsulfated resveratrol degraded ~50% during 24 h under these conditions.[40] In another study, epicatechin persulfate was found to be stable under neutral and basic conditions and considerably unstable under acidic conditions.[41] We have previously proved the human plasma stability for polysulfated flavonoids *O*-rutinosides.[13] In this work, we assessed not only the stability in human plasma but also the chemical stability in aqueous solution at different storage temperatures in a range of concentrations and pH values for the polysulfated xanthone *C*-glucoside **7** (Figures 3 and 4).

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Concerning stability in aqueous solutions, compound **7** was found to be chemically stable for 15 days at several storage conditions, room temperature, 4 °C, and −20 °C, at high, low, and medium concentrations (Figure 3), i.e., the concentration detected on each day was not significantly different from the initial one. Furthermore, compound **7** was highly soluble in water and did not produce crystals when stored at 4 °C or −20 °C. Compound **7** did not degrade at the range of pH values selected to mimic the in vivo oral dosing (Figure 4), i.e., the % of compound **7** remaining at 120 min was higher than 85%.[42] Finally, compound **7** was found stable after 3 h at 37 °C in human plasma, i.e., the concentration after 3 h was not significantly different from the initial one. This was consistent with the plasma stability previously reported for diosmin hexasulfate (**1**)[13,43] and rutin decasulfate (**4**).[13]

From the overall results, it can be concluded that compound **7**, highly soluble in water, is stable either in water or in plasma.

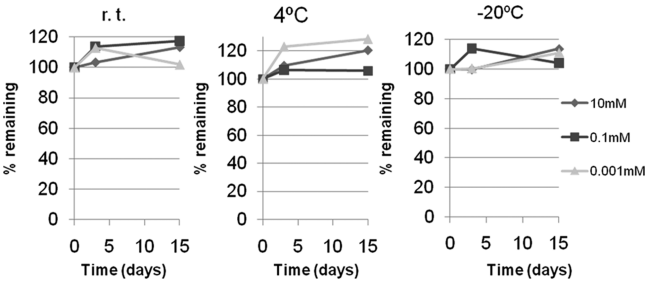

Figure 3. Stability of compound **7** in aqueous solutions at storage conditions at room temperature, 4 °C, and −20 °C.

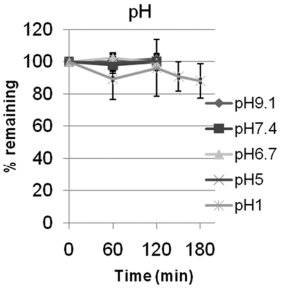

Figure 4. Stability−pH profile for compound **7** (1.00×10^{-5} M).

RESULTS AND DISCUSSION

The anticoagulant activity of the sulfated molecules **6**, **7**, **10**, and **13** was screened in vitro in human plasma by activated partial thromboplastin (APTT), prothrombin (PT), and thrombin time (TT) assays. The nonsulfated parent compounds **5** and **12** and sodium sulfate were also considered in order to explore the influence of the sulfate group on the anticoagulant activity.

The results for the sulfated derivatives tested (**6**, **7**, **10**, and **13**) on APTT, PT, and TT are summarized in Figure 5. For the most potent compounds the concentration needed to double the coagulation times ($APTT_2$, PT_2, TT_2) was calculated (Table 1). The nonsulfated compounds **5** and **12** and sodium sulfate solution 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).

The investigated polysulfated xanthonosides **6**, **7**, and **13** showed anticoagulant properties in a dose dependent manner in all clotting times (Figure 5). The nonglycosylated xanthone disulfate **10** was only active, in PT and TT, at the highest concentration tested (5.00×10^{-3} M).

Prolongation of APTT and PT was observed for all the sulfated xanthones (**6**, **7**, **10**, and **13**). Polysulfated xanthones **6**, **7**, and **13** were able to completely block these clot formation pathways at high concentrations (Figure 5). Xanthone **13** was the most potent compound ($APTT_2 = 6.4 \times 10^{-5}$ M, $PT_2 = 7.1 \times 10^{-5}$ M). Sulfated compound **10** only increased the APTT and PT approximately 1.3 and 1.1 times, respectively, at 5.00×10^{-3} M.

The TT was sensitive to the presence of compounds **6**, **7**, and **13**, which were able to completely prevent thrombin activation, at the highest concentration tested (Figure 5).

Table 1. Concentrations Needed To Double the Clotting Times[a] for Sulfated Xanthones 6, 7, 10, and 13

compd	$APTT_2$	PT_2	TT_2
6	0.39	nd	nd
7	0.21	1.60	1.96
10	>5	>5	na
13	0.06	0.71	0.61

[a]Values in concentrations expressed in 10^{-3} M represent the average of three independent experiments with a standard deviation of <10%. Positive control, heparin: $APTT_2 = 0.4$ U/mL, $PT_2 = 1.9$ U/mL, and $TT_2 = 0.2$ U/mL. nd: not determined. na: not active at 5.00×10^{-3} M ($P > 0.05$).

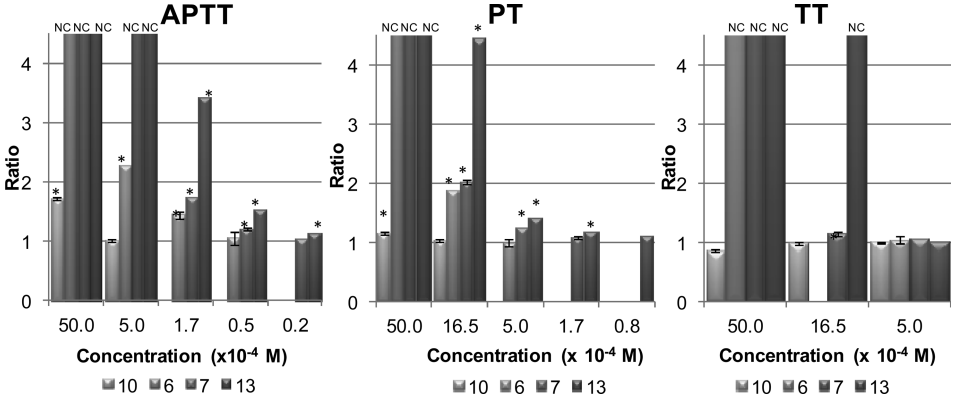

Figure 5. Effects of sulfated derivatives (**6**, **7**, **10**, and **13**) on APTT, PT, and TT clotting assays using human pooled plasma, expressed as ratio of clotting time in the presence/absence of compound. Data points represent the average of three experiments performed in duplicate with a standard deviation of <10%. NC, no coagulation: clotting time values greater than 165 s (PT and TT) and 110 s (APTT). ∗, $P < 0.05$.

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Table 2. In Vitro Inhibitory Activity[a] of Polysulfated Xanthones 7 and 13 against Human FXa and ATIII Inhibition of FXa in the Presence of CBS31.39 (1.25×10^{-4} M)

	human FXa		ATIII/FXa	
compd	% inhib[b]	IC_{50} ($\times 10^{-4}$ M)	% inhib[b]	IC_{50} ($\times 10^{-4}$ M)
7	52.10 ± 2.67	9.70 ± 0.02	41.0 ± 6.00	nd
13	66.26 ± 2.13	7.40 ± 0.05	88.82 ± 5.30	1.90 ± 0.03

[a] Each value represents the average ± SEM of three independent experiments done in duplicate. [b] Results expressed as percentage of inhibition at 1.00×10^{-3} M. The % of rivaroxaban FXa inhibition at 5.00×10^{-9} M was equal to 52.00 ± 3.62 (IC_{50} = (4.59 ± 1.63) × 10^{-9} M). The % of LMWH ATIII/FXa inhibition at 0.20 U/mL was equal to 97.96 ± 0.58.

In summary, all the sulfated compounds (**6**, **7**, **10**, and **13**) prolonged APTT and PT, and compounds **6**, **7**, and **13** also prolonged TT pathway. As the three clotting assays recorded interactions at different stages of the coagulation process, preliminary information about the mode of action can be obtained. As the sulfated compounds **6**, **7**, and **13** prolonged APTT, PT (APTT more than PT), and TT, their mechanism of action may be implicated in the common pathway of the coagulation cascade.

A structure−activity relationship can be recognized concerning the anticoagulant action and the number of sulfate groups, i.e., the anticoagulant effects increased with the increase of the number of sulfates. Xanthone 3,6-β-D-glucopyranoside persulfate (**13**), with eight sulfate groups, was more potent than heptasulfated mangiferin **7**, and compound **7** was more potent than tetrasulfated compound **6**. The disulfated xanthone **10** was only slightly active in prolonging the clotting times. Nevertheless, by comparison of these results with the earlier obtained for polysulfated flavonosides,[13] xanthone **13** with eight sulfate groups ($APTT_2 = 6.4 \times 10^{-5}$ M) was not significantly different from decasulfated flavonoid rutin (**4**, $APTT_2 = 6.6 \times 10^{-5}$ M) and xanthone **6** ($APTT_2 = 3.9 \times 10^{-4}$ M) with only four sulfate groups had the same order of potency as hexasulfated diosmin (**1**, $APTT_2 = 4.1 \times 10^{-4}$ M) and hesperidin (**2**, $APTT_2 = 4.4 \times 10^{-4}$ M).

Compounds **7** and **13** were further evaluated for selectivity against the critical enzymes targeted by current anticoagulant therapy: thrombin, FXa, and ATIII. Inhibition of thrombin and FXa, in the presence and 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 was compared with that obtained in the absence of the compound. Reference inhibitors rivaroxaban (direct FXa inhibitor), LMWH (ATIII/FXa activator), and PPACK (direct thrombin inhibitor) were also tested in the same conditions. Kinetic studies of the inhibitory activity of sulfated xanthones **7** and **13** against FXa were fitted by the Michaelis−Menten equation (nonlinear regression analysis, data not shown).

Both xanthones showed a direct inhibitory effect on FXa (Table 2), with compound **13** exhibiting higher potency than compound **7**. For compound **13** this effect was significantly ($P < 0.05$) enhanced by the presence of ATIII, suggesting also an indirect action on FXa by activation of ATIII. For compound **7** the percentage of FXa inhibition in the presence of ATIII was not significantly different from that obtained in its absence ($P > 0.05$). Therefore, mangiferin 2′,3,3′,4′,6,6′,7-heptasulfate (**7**) was found to be a direct FXa inhibitor and persulfated 3,6-(*O*-β-glucopyranosyl)xanthone (**13**) a dual direct and indirect FXa inhibitor. At 1.00×10^{-3} M compound **13** (FXa inhibition of 66%) and even compound **7** (FXa inhibition of 52%) were more potent on FXa than the earlier obtained direct FXa inhibitors ethoxyrutin nonasulfate (**3**, FXa inhibition of 39%) and rutin persulfate (**4**, FXa inhibition of 32%).[13] By comparison of the indirect FXa inhibition results, compound **13** (ATIII/FXa inhibition of 89%) was also more active than the earlier obtained ATIII/FXa inhibitors diosmin hexasulfate (**1**, ATIII/FXa inhibition of 39%) and hesperidin hexasulfate (**2**, ATIII/FXa inhibition of 46%).[13] The Michaelis−Menten fit for both xanthones **7** and **13** suggests a noncompetitive inhibition against FXa (data not shown), which is in accordance with previously reported data for some sulfated benzofuran small molecules.[44] Dual direct and indirect FXa inhibitors, like compound **13**, or direct FXa inhibitors, like compound **7**, have advantages regarding heparins or fondaparinux primarily because they can inhibit both circulating and clot-bound enzymes. Indirect FXa inhibitors require ATIII as a cofactor and do not inhibit FXa bound to the prothrombinase complex. In contrast, direct FXa inhibitors directly engage the active center of the FXa molecule and thus inhibit the free and the attached FXa to the prothrombinase complex.[45]

None of the compounds **7** 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), which highlights their FXa selectivity. FXa activates clotting over a wider concentration range compared to thrombin, in both in vitro and in vivo systems.[46] Consequently, FXa inhibitors compared with thrombin inhibitors are expected to have a wider therapeutic window and thus to reduce the need for coagulation monitoring. Because of the absence of effect in the amidolytic compared to the coagulation assay, the action in the TT assay for compounds **7** and **13** does not seem not to be the result of an inhibition of thrombin but instead mediated by an interaction with the thrombin action on fibrinogen or fibrinogen itself, which cannot be measured in the chromogenic substrate assay.

To assess if the anticoagulant effect of sulfated xanthones **7** and **13** could also interfere with the protein C pathway, the determination of activated protein C (APC) in citrated human plasma was evaluated by a functional test. Protein C was activated by a specific activator derived from the venom of *Agkistrodon c. contortrix*, and the quantity of enzyme thus formed was measured by its amidasic activity on the synthetic substrate CBS 42.46.

At 2.50×10^{-4} M, compounds **7** and **13** had no significant influence in the protein C activity (Figure 6). Only at high concentrations (2.00×10^{-3} M), compounds **7** and **13** decreased the APC to pathological levels (less than 50%, Figure 6). Nevertheless, compounds **7** and **13**, at the concentration that led to approximately 40% of APC inhibition, also showed deeply decreased prothrombin levels (Figure 6). Since low levels of prothrombin were observed, the risk of thrombosis due to the APC inhibition for these compounds is not expected, as it happens for heparin and oral anticoagulants.[47−50]

To have a global picture of the anticoagulant effect of sulfated molecules **7** and **13** and to warrant anticoagulation activity in whole blood, thromboelastography (TEG) analyses were carried out. Classical clotting tests are performed in citrated plasma and provide information about only one isolated segment of the coagulation system (secondary hemostasis). The clot quality and the impact of platelets on coagulation are not assessed by these assays. In contrast, TEG is performed in whole blood and the clot

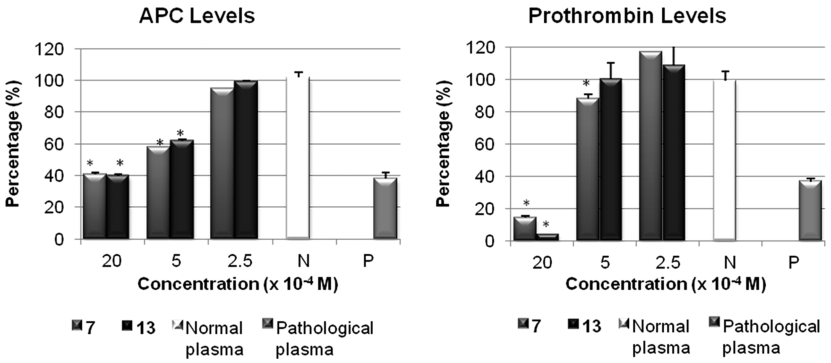

Figure 6. Dose-dependent effect of compounds **7** and **13** on activated protein C (APC) and prothrombin levels. Data points represent the average ± SD of three independent experiments done in duplicate. ∗, $P < 0.05$.

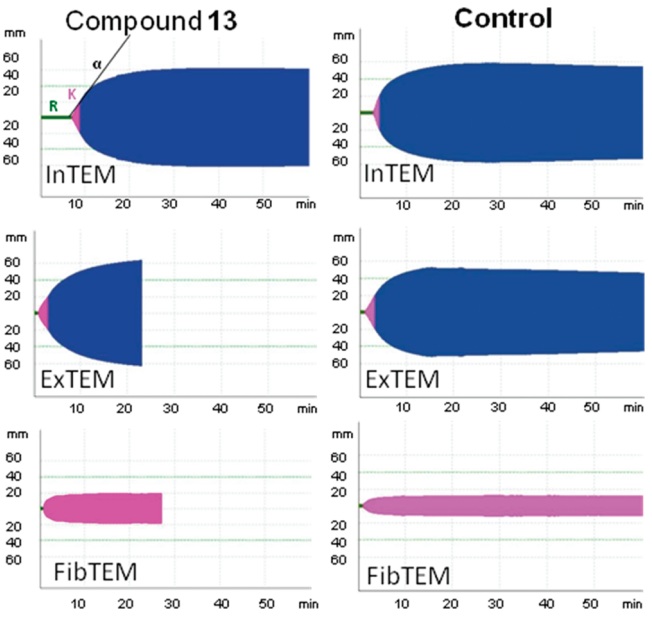

Figure 7. Thromboelastography (TEG) representative tracings of compound **13** (3.12×10^{-4} M) and control (saline).

is induced under a low shear environment resembling an in vivo sluggish venous flow. TEG displays qualitatively two distinct parts of hemostasis (the part that produces the clot and the part that causes the breakdown of the clot) and allows the acquisition of quantitative information about the kinetics of clot formation and growth as well as the clot strength and stability attained by clots.[51] The anticoagulant effects of sulfated compounds **7** and **13** in whole human blood were evaluated at 3.12×10^{-4} M. Modified thromboelastograms were obtained (Figure 7, e.g., compound **13**) by adding to the whole blood an intrinsic activator (InTEM) or an extrinsic activator (ExTEM). It was also possible to eliminate platelet contribution from the TEG sample and study the fibrin part of the clot by adding cytochalasin D and Ca^{2+} (FibTEM). The parameters obtained from the InTEM thromboelastogram were *R*, the period of time from initiation of the test to the initial fibrin formation (clotting time); *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 buildup and cross-linking (clot formation and growth); and A_{10}, the amplitude of the clot at 10 min (Table 3).

Table 3. Effects[a] of Polysulfated Xanthones 7 and 13 (3.12×10^{-4} M) in Whole Human Blood on the Parameters Obtained from InTEM-Thromboelastography

	InTEM			
compd	R (s)[b]	K (s)[c]	∝ (deg)[d]	A_{10} (mm)[e]
blood (saline)	188	82	77	57
7	247*[f]	86	73	50
13	469*[f]	114*[f]	68*[f]	51*[f]

[a] Each value represents the average of two independent experiments. [b] *R*, clotting time expressed in seconds (s). [c] *K*, clotting formation time expressed in seconds (s). [d] ∝, angle expressed in degrees (deg). [e] A_{10}, amplitude expressed in mm at 10 min. [f] ∗, $P < 0.05$.

In the presence of compounds **7** and **13**, abnormal thromboelastograms were obtained when an intrinsic activator was added in InTEM test (Table 3), an effect similar to that observed in the presence of a factor deficiency (anticoagulants or hemophilia).[52] Compared to control values, compound **7** increased only the clotting time (*R*), while compound **13** increased also the clotting formation time (*K*) and decreases ∝ and A_{10} (Table 3). This indicates that the kinetics of fibrin polymerization and networking and the strength of the formed clot, which is highly dependent on platelets, are significantly retarded by the presence of sulfated compound **13**, while compound **7** is only affecting the clotting factors. Nevertheless, normal thromboelastograms were obtained in the presence of compounds **7** and **13** when the platelet-specific effect on A_{10} of TEG tracings is eliminated (FibTEM test, Figure 7). These results allowed the conclusion that an action on platelets would also be implicated in the mode of action of compounds **7** and **13**.

The overall TEG results showed where the imbalance of the hemostasis system was located. In the presence of compounds **7** and **13**, there were no signals of fibrinolysis. The blood became hypocoagulable in the presence of both compounds. The enzymatic reactions were decreased, as shown by the *R* parameter. There was a low fibrinogen level as depicted by the ∝ parameter, and the platelet function was decreased, as represented by the A_{10} parameter.

In vivo studies in mice were also performed for compound **7** to preliminarily assess its anticoagulant and toxicity effects. To estimate the in vivo anticoagulant activity, APTT, PT, and TT were determined after 150 and 300 μmol/kg intraperitoneal (ip) administration or at 120 min after 300 μmol/kg of oral administration by gavage. These values were compared with the APTT,

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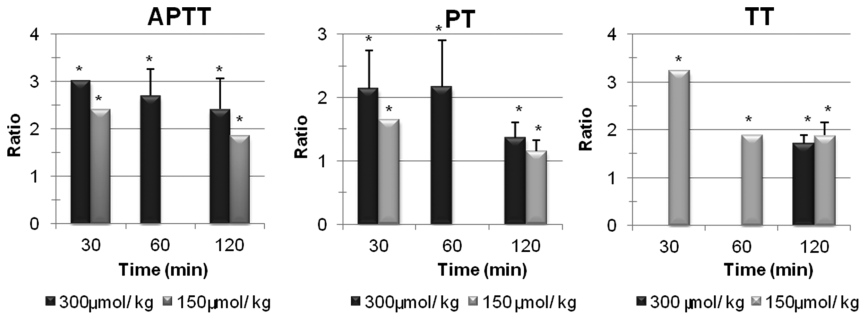

Figure 8. Dose-dependent APTT, PT, and TT prolonging activities of compound **7** after ip administration in mice, expressed as ratio of the clotting time of compound-treated mice to clotting time of saline-treated mice (control group). Data points represent the average ± SEM of three independent experiments. ∗, $P < 0.05$.

Table 4. Antiaggregant Effects[a] of Nonsulfated (5 and 12) and Sulfated (7 and 13) Xanthones (6.25×10^{-4} M) on Whole Human Blood Evaluated with PFA-100 and Multiplate Analyzers

	PFA-100 closure time (s)		multiplate AUC (U)[b]				
whole blood	Col/ADP	Col/EPI	TRAP test	ASPI test	Col test	ADP test	ADP-HS test
ref values	71−118	94−193	84−128	71−115	72−125	57−113	43−100
blood (saline)	119	168	106	72	60	72	46
Na_2SO_4	nd	nd	131	87	84	78	47
ASA	nd	nd	104	19*[c]	49*[c]	77	54
5	nd	nd	115	72	77	65	44
12	nd	nd	110	84	66	68	43
7	146*[c]	165	112	44*[c]	52*[c]	31*[c]	30*[c]
13	174*[c]	268*[c]	91	29*[c]	44*[c]	25*[c]	22*[c]

[a]Each value represents the average of two independent experiments done in duplicate. nd: not determined. [b]Area under the aggregation curve (AUC) expressed in U (1 U = 10 AU × min). [c]∗, $P < 0.05$.

PT, and TT values obtained with the saline-treated mice group and expressed as a ratio (Figure 8). Plasma levels of glutamic−oxaloacetic transaminase/aspartate aminotransferase (GOT/AST) and glutamate−pyruvate−transaminase/alanine aminotransferase (GPT/ALT) were determined after the ip administration of these molecules.

After 30 min of ip administration, compound **7** was highly active, increasing the APTT to the desirable therapeutic range (1.5−2.3 times the mean of the normal APTT range,[53] Figure 8). The rapid onset of action revealed by compound **7** is a desirable feature compared to oral anticoagulants drugs acenocoumarol and warfarin sodium. Moreover, at both concentrations compound **7** was still active after 120 min; it is reasonable to speculate that a desirable duration of action will be sustained.[53] After oral administration, compound **7** was not active (results not shown). These results are in accordance with the results previously obtained for polysulfated flavonosides.[13]

After ip administration of compound **7** at 300 μmol/kg, GOT and GPT plasma levels were not significantly different (125 and 46 U/L, respectively) from those of saline treated mice (108 and 40 U/L, respectively) ($P > 0.05$). These preliminary results, obtained after a single acute dose, suggest that compound **7** has low hepatotoxic potential, in contrast to unfractionated and LMW heparins.[3,54,55]

After obtaining the in vivo results, we decided to further investigate the effects of sulfated derivatives (**7** and **13**) on platelets. Thus, the platelet aggregation in whole blood in the presence of sulfated xanthones **7** and **13** (Table 4, Figure 9) was evaluated by measuring the collagen/adenosine 5′-diphosphate (Col/ADP) and collagen/epinephrine (Col/EPI) closure times and impedance platelet aggregometry induced by thrombin receptor-activating peptide (TRAP test), arachidonic acid (ASPI test), collagen (Col test), ADP (ADP test), and ADP and prostaglandin E1 (ADP-HS test). The parent nonsulfated compounds **5** and **12**, a highly ionic salt (sodium sulfate), and acetylsalicylic acid (ASA, cyclooxygenase-1 inhibitor) were also tested in the same conditions.

The Col/EPI test cartridge is the primary cartridge used to detect platelet dysfunction induced by intrinsic platelet defects, von Willebrand disease, or exposure to platelet inhibiting agents. The Col/ADP test cartridge is used to indicate if abnormal results obtained with Col/EPI test cartridge may have been caused by the effect of ASA. Usually, a pattern of abnormal Col/EPI and normal Col/ADP is observed with ASA ingestion cases. While ASA has been shown to increase the Col/EPI closure time,[56] clopidogrel has been shown to increase the Col/ADP closure time.[57] Closure times do not vary with heparin administration.[57] Considering compounds **7** and **13**, PFA-100 closure times of both Col/ADP and Col/EPI cartridges were markedly longer with xanthone **13**, and only the Col/ADP closure time was increased with xanthone **7** at 6.25×10^{-4} M (Table 4).

None of the investigated compounds affected the TRAP test, which is affected by GpIIb/IIIa antagonists and thrombin inhibitors The ASPI test specifically measures the activation by arachidonic acid, the substrate of cyclooxygenase 1. Cyclooxygenase forms thromboxane A2, which is a potent platelet activator. When

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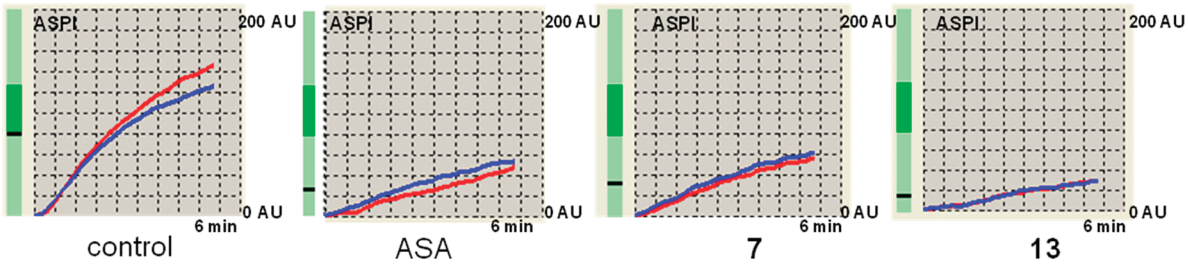

Figure 9. Multiplate traces in ASPI test. During a measurement period of 6 min the change in electrical impedance is calculated from the mean values of the two curves: control, saline; ASA, acetylsalicylic acid (final concentration, 6.25×10^{-4} M); **7** and **13**, sulfated compounds (final concentration, 6.25×10^{-4} M).

the platelet cyclooxygenase is blocked, the formation of thromboxane A2 is inhibited and no alteration or minor platelet activation is recorded. In the Col test, collagen activates the collagen receptor, which leads to a release of endogenous arachidonic acid. The addition of xanthones **7** and **13** into the blood led to a reduced aggregation response in both the ASPI test (Table 4, Figure 9) and the Col test (Table 4).

The results obtained in the ASPI and Col tests in the presence of xanthones **7** and **13** (Table 4), when compared with blank and ASA, revealed their ability to disrupt the arachidonic acid pathway. The ADP test is sensitive to ADP receptor inhibitors, and because of the addition of prostaglandin E1, the ADP-HS test has even an increased sensitivity toward these inhibitors. Both sulfated xanthones (**7** and **13**) also affected the ADP and ADP-HS tests (Table 4), in contrast to ASA. Dual antiplatelet therapy with ASA and a thienopyridine is often recommended for high-risk patients.[58] ASA has only a modest effect, preventing cardiovascular events in approximately 25% of patients with atherosclerosis.[58] The thienopyridines (clopidogrel, ticlopidine) are only slightly more effective.[58] The modest efficacy of these drugs may be related to the fact that there are multiple alternative pathways for platelet activation. The different antiaggregant behavior of these small sulfated molecules affords an opportunity to discover different molecules from the known antiplatelet agents with possibly different pharmacological profile. Furthermore, molecules such as xanthones **7** and **13**, which simultaneously target the antiplatelet and anticoagulant pathways, could be useful to prevent and treat both venous and arterial thrombosis. In the past 5 years, dual anticoagulant/antiplatelet agents have already been described,[59−61] although such dual agents have not yet achieved clinical development. From the overall results and putative in vivo efficacy, with no suggestion of acute side effects, xanthones **7** and **13** show potential to lead a new class of therapeutic agents in the prevention and treatment of thromboembolic events.[62]

CONCLUSION

The sulfation as a molecular modification of the xanthonoid scaffold was a successful strategy to obtain analogues of polysulfated flavonosides with higher anticoagulant potency. Two polysulfated xanthonosides proved to be dual acting inhibitors of thrombosis, combining anticoagulant and antiplatelet effects into a single molecule. Both showed antiplatelet activity by inhibition of arachidonic acid and ADP-induced platelet aggregation, while concerning the anticoagulant mechanism of action, heptasulfated mangiferin (**7**) was found to be a direct FXa inhibitor and persulfated diglycosidic xanthone (**13**) a dual direct and indirect FXa inhibitor.

We believe that in contrast to unfractionated and LMW heparins, polysulfated xanthonosides represent a new generation of cardiovascular agents, with a defined composition and feasible synthesis. Additionally, the less charge density allows a systemic anticoagulant action after intraperitoneal administration, with these compounds not likely to induce hemorrhagic complications or hepatic toxicity.

EXPERIMENTAL SECTION

Chemistry. Triethylamine−sulfur trioxide adduct (S 5139) was purchased from Fluka (Spain), and mangiferin (**5**, M 3547), 2,2′,4,4′-tetrahydroxybenzophenone (**8**, T16403), 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl bromide (A1750), and sodium methoxide 0.5 M in methanol (71751) were purchased from Sigma-Aldrich (Spain). The solvents used were products pro analysis or HPLC grade of the firms Sigma-Aldrich and Fluka. Spectra/Por dialysis membrane, MWCO 1000, was purchased from Spectrum Laboratories, Inc. (CA, U.S.). MW reactions were performed in sealed reaction vessels (30 mL) using a MicroSYNTH 1600 synthesizer from Millestone (ThermoUnicam, Portugal). TLC was performed using Polygram cel 400 UV254 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 spectrophotometer (software WinFirst, version 2.10), 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 MHz for ^{13}C). Chemical shifts are expressed as 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). HRMS mass spectra were measured on a APEX III mass spectrometer, recorded as ESI (electrospray ionization) mode in Centro de Apoio Científico e Tecnolóxico á Investigation (CACTI, University of Vigo, Spain). The HPLC analyses were carried out on a system SMI pump series II (Gloucester, U.K.) 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, U.S.). The mobile phases were degassed for 15 min in an ultrasonic bath before use. The purity of each compound was determined by HPLC-DAD analysis.[13] All tested compounds, whether synthesized or purchased, possessed a purity of at least 95%.

General Procedure for Sulfation of Mangiferin (5). To a solution of mangiferin (**5**, 1 mmol) in DMA (15 mL), triethylamine−sulfur trioxide adduct (4 equiv/OH led to compound **6**; 8 equiv/OH led to compound **7**) was added, and the suspension was heated at 65 °C for 24 h. The mixture was poured into acetone (150 mL) under basic

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conditions (a few milliliters 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. The solid obtained was further purified from other salts (monitored by IR) using a Spectra/Por 6 regenerated cellulose MWCO 1000.

Mangiferin 2″,3″,4″,6″-*O*-Tetrasulfate (6). Brown solid (0.183 g, 0.22 mmol, 22%), mp >340 °C (water). IR (KBr) υ_{max}: 3600−3400, 1622, 1258, 1127, 1067, 804, 616. ^{1}H NMR (DMSO-d_6, 300.13 MHz) δ: 13.67 (1H, s, 1-O*H*), 10.70 (1H, brs, 6-O*H*), 10.40 (1H, brs, 7-O*H*), 9.86 (1H, brs, 3-O*H*), 7.39 (1H, s, H-8), 6.87 (1H, s, H-5), 6.32 (1H, s, H-4), 5.30−5.20 (1H, m, H-3′), 4.85 (1H, d, J = 5.5 Hz, H-1′), 4.77 (1H, brt, H-2′), 4.49 (1H, m, H-4′), 4.12 (1H, m, H-5′), under water (2H, H-6′). ^{13}C NMR (DMSO-d_6, 75.47 MHz) δ: 179.1 (C-9), 169.7 (C-3), 161.5 (C-1), 156.2 (C-4a), 154.0 (C-6), 150.5 (C-10a), 143.6 (C-7), 111.7 (C-8a), 108.1 (C-8), 107.5 (C-2), 102.6 (C-5), 101.2 (C-9a), 93.3 (C-4), 79.7 (C-5′), 76.4 (C-2′), 74.4 (C-3′), 71.5 (C-4′), 70.2 (C-6′), 68.3 (C-1′).

Mangiferin 2′,3,3′,4′,6,6′,7-*O*-Heptasulfate (7). Orange solid (1.04 g, 0.93 mmol, 93%), mp >340 °C (water). IR (KBr) υ_{max}: 3600−3400, 1614, 1251, 1117, 1028, 804, 600. ^{1}H NMR (DMSO-d_6, 300.13 MHz) δ: 13.43 (1H, s, 1-O*H*), 8.26 (1H, s, H-8), 7.71 (1H, s, H-5), 7.23 (1H, s, H-4), 5.39 (1H, t, J = 7.4 Hz, H-3′), 4.77 (1H, brt, H-2′), 4.76 (1H, brd, H-1′), 4.73 (1H, m, H-4′), 4.09 (1H, d, J = 9.9 Hz, H-6′a), 3.90 (1H, m, H-5′), 3.76 (1H, t, J = 9.6 Hz, H-6′b). ^{13}C NMR (DMSO-d_6, 75.47 MHz) δ: 180.0 (C-9), 162.0 (C-1), 159.0 (C-3), 155.8 (C-4a), 151.5 (C-6), 150.9 (C-10a), 140.9 (C-7), 113.9 (C-8), 113.4 (C-8a), 109.7 (C-2), 106.3 (C-5), 103.3 (C-9a), 96.4 (C-4), 79.4 (C-5′), 77.6 (C-1′), 73.3 (C-3′), 72.3 (C-2′), 69.5 (C-4′), 67.6 (C-6′). HRMS (ESI$^+$) m/z calcd for $C_{19}H_{11}O_{32}S_7Na_8$ 1158.645 45, found 1158.644 46.

Synthesis of 3,6-Dihydroxyxanthone (9). 3,6-Dihydroxyxanthone (**9**, 1.8 g, 7.9 mmol, 98%) was obtained from 2,2′,4,4′-tetrahydroxybenzophenone (**8**, 2 g, 8.1 mmol) after 24 h in the furnace (220 °C).[38]

Synthesis of 3,6-(*O*-β-Glucopyranosyl)xanthone (12). TBAHS (3 g, 8.8 mmol, 2 equiv/OH) and 3,6-dihydroxyxanthone (**9**, 0.5 g, 2.2 mmol) were mixed in $CHCl_3$ (20 mL) at 50 °C. 2,3,4,6-Tetra-*O*-acetyl-α-D-glucopyranosyl bromide (1.8 g, 4.4 mmol, 1 equiv/OH) was added to the suspension followed by 0.15 M aqueous K_2CO_3 (100 mL, 15 mmol) and left to react during 36 h. After cooling, the reaction mixture was filtered and the solid washed with dichloromethane. The filtrate was concentrated, alkalinized by adding aqueous sodium bicarbonate solution, and extracted with dichloromethane (3 × 30 mL). The organic phases were collected, dried over $MgSO_4$ and the solvent was removed under reduced pressure to afford compound **11** (1.2 g). Compound **11** was treated with 0.5 M sodium methoxide (35 mL, 17.5 mmol, 2 mol/glucose) in 220 mL of methanol (extensively dried over activated 4 Å molecular sieves) and left stirring at room temperature overnight. The reaction solution was further purified by solid phase extraction (silica flash cartidges GraceResolv). The acetone−methanol (80:20) fractions were collected and the solvent was evaporated under reduced pressure to afford a brown solid corresponding to compound **12** (0.61 g, 1.1 mmol, 50%). IR (KBr) υ_{max}: 3600−3400, 1625, 1440, 1263, 1065, 595, 489. ^{1}H NMR (DMSO-d_6, 500.13 MHz) δ: 8.10 (2H, d, J = 8.8 Hz, H-1, H-8), 7.22 (2H, d, J = 2.2 Hz, H-4, H-5), 7.11 (2H, dd, J = 8.8 and 2.2 Hz, H-2, H-7), 5.51 (1H, brs, 3′-O*H*), 5.26 (1H, brs, 4′-O*H*), 5.18 (1H, d, J = 8.8 Hz, H-1′), 4.71 (1H, brs, 6′-O*H*), 3.69 (1H, m, H-6′a), 3.50 (1H, under water, H-6′b), 3.46 (1H, under water, H-5′), 3.35 (1H, t, J = 8.8 Hz, H-3′), 3.30 (1H, t, J = 8.3 Hz, H-2′), 3.20 (1H, t, J = 8.8 Hz, H-4′). ^{13}C NMR (DMSO-d_6, 75.47 MHz) δ: 174.3 (C-9), 162.5 (C-3, C-6), 157.4 (C-4a, C-10a), 127.6 (C-1, C-8), 115.8 (C-8a, C-9a), 114.6 (C-2, C-7), 103.1 (C-4, C-5), 99.8 (C-1′), 77.2 (C-5′), 76.4 (C-3′), 73.2 (C-2′), 69.6 (C-4′), 60.7 (C-6′). HRMS (ESI$^+$) m/z calcd for $C_{25}H_{28}O_{14}Na$ 575.137 13, found 575.137 00.

Synthesis of 3,6-(*O*-β-Glucopyranosyl)xanthone Persulfate (13). To a solution of the xanthone glycoside **12** (0.53 g, 0.97 mmol) in DMA (10 mL), triethylamine−sulfur trioxide adduct (7.9 g, 6 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 to 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 milliliters 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. The solid obtained was further purified from other salts (monitored by IR) using a Spectra/Por 6 regenerated cellulose MWCO 1000 (**13**, 0.8 g, 60%). IR (KBr) υ_{max}: 3600−3400, 1614, 1251, 1104, 1027, 799, 612. ^{1}H NMR (DMSO-d_6, 300.13 MHz) δ: 8.11 (2H, d, J = 8.9 Hz, H-1, H-8), 7.14 (2H, d, J = 2.1 Hz, H-4, H-5), 7.10 (2H, dd, J = 8.9 and 2.1 Hz, H-2, H-7), 5.64 (1H, d, J = 3.7 Hz, H-1′), 4.65 (1H, dd, J = 8.3 and 2.9 Hz, H-6′a), 4.44 (1H, t, J = 2.9 Hz, H-6′b), 4.36−4.31 (1H, m, H-5′), 4.05 (1H, brt, H-3′), 4.01 (1H, brt, H-2′), 3.90 (1H, t, J = 9.5 Hz, H-4′). ^{13}C NMR (DMSO-d_6, 75.47 MHz) δ: 174.3 (C-9), 162.5 (C-3, C-6), 157.2 (C-4a, C-10a), 127.7 (C-1, C-8), 115.9 (C-8a, C-9a), 113.9 (C-2, C-7), 103.8 (C-4, C-5), 98.1 (C-1′), 75.4 (C-5′), 74.2 (C-3′), 71.0 (C-2′), 67.1 (C-4′), 62.0 (C-6′). HRMS (ESI$^+$) m/z calcd for $C_{25}H_{20}O_{38}S_8Na_9$ 1390.647 20, found 1390.654 06.

Stability Studies. Human Plasma. The plasma stability of compound **7**, at 2.50 × 10^{-5} M final concentration, was measured 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 Eppendorf tubes on a bath shaker at 37 °C for 180 min. The reaction was quenched with 4× cold acetonitrile (HPLC grade), followed immediately by mixing and centrifugation during 15 min at 14 000 rpm. The time zero samples were quenched immediately after the sample was added to plasma. After filtration (Millipore), the supernatant was directly transferred to HPLC vials and analyzed by HPLC. Five pH values (1, 5, 6.7, 7.4, 9.1) were selected. The following buffers HCl (pH 1.0), sodium acetate (0.05 M, pH 5.0), potassium phosphate (0.1 M, pH 6.8), PBS (pH 7.4), and sodium boric acid (0.05 M, pH 9.1) were prepared. Then 20 μL of water stock compound solution was added to 1980 μL of each buffer solution to obtain a final test compound concentration of 1 × 10^{-5} M. The samples were incubated in a temperature-controlled autosampler at 37 °C. The HPLC autosampler was programmed to inject samples periodically at 0, 60, 120, and 180 min. The experiments were run at three different concentrations of water solutions (1.00 × 10^{-2}, 1.00 × 10^{-4}, 1.00 × 10^{-6} M). The stability challenged samples were allowed to sit 1, 7, and 15 days at laboratory temperature, 4 °C, and −20 °C. For HPLC chromatographic conditions, the mobile phase used for chemical and plasma stability studies of compound **7** was 50 mM sodium acetate buffer (adjusted to pH 4.5 with acetic acid, HPLC grade) and acetonitrile (90:10) at a constant flow rate of 1.0 mL/min.

Biological Activity. Clotting Assays. Human blood was obtained from 10 healthy donors 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. Compounds **6**, **7**, **10**, **12**, and **13** were dissolved in water, and compound **5** was dissolved in water with 10% DMSO. The final concentration of sulfated compounds (**6**, **7**,

10, and **13**) in these assays ranged from 5.00×10^{-3} to 5.00×10^{-5} M. Only a single concentration was tested for nonsulfated compounds **5** and **12** and sodium sulfate solution (5.00×10^{-3} M). Heparin (0.05−5 UI/mL) was used as positive control. In the control group, water (control for compounds **6**, **7**, **10**, **12**, and **13**) or 10% DMSO (control for compound **5**) was used. APTT, PT, and TT tests were performed using an ACL100 coagulometer (IZASA, Portugal). The following commercial kits were used: no. 0020006800 (HemosIL, SynthASil, IZASA, Portugal) for the APTT, no. 0008469810 (HemosIL, PT-fibrinogen, IZASA, Portugal) for the PT, and no. 0009758515 (HemosIL, thrombin time, IZASA, Portugal) for the TT. The assays were carried out as previously reported.[13] 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. For clotting time values greater than 165 s (PT and TT) and 110 s (APTT), values of 165 and 110 s, respectively, were arbitrarily assigned for statistical analysis.

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 U/mL). The chromogenic substrates used were Chromozym TH (tosyl-Gly-Pro-Arg-*p*-nitroaniline (pNA), Roche Applied Science) for thrombin and CBS 31.39 (CH_3SO_2-D-Leu-Gly-Arg-pNA, AcOH; Diagnostica Stago) for FXa. Stock solutions of the enzymes were obtained according to the manufacturers' 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, 0.06, and 1 U/mL for FXa, thrombin, and ATIII, respectively. The final substrate concentrations in the reactions were 178 and 125 μM (in water) for Chromozym TH and CBS31.39, respectively. For the IC_{50} determination of CBS 31.39 hydrolysis by FXa, various concentrations of the inhibitors **7** and **13** were investigated (1.00×10^{-4}, 2.00×10^{-4}, 4.00×10^{-4}, 8.00×10^{-4}, and 1.00×10^{-3} M). For the kinetics studies, various concentrations of the sulfated inhibitors (5.00×10^{-4} to 1.00×10^{-3} M) and CBS 31.39 (1.25×10^{-4} and 2.50×10^{-4} M) were used. Under the experimental conditions, less than 10% of the substrate was consumed in all assays [ε_{405nm}(*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, VT, U.S.) as previous reported.[13] Test compounds were diluted in water. Reference inhibitors rivaroxaban (direct FXa inhibitor), PPACK (direct thrombin inhibitor, D-Phe-L-Pro-L-Arg-CMK), and LMWH (ATIII/FXa activator) were assayed in the same conditions.

Activated Protein C Levels. APC was measured after addition of compound solution (30 μL, saline) to citrated human plasma (300 μL) using a chromogenic assay (STA protein C chromogen 6007935, Diagnostica Stago, Roche, Portugal). Protein C was activated by a specific activator derived from the venom of *Agkistrodon c. contortrix* (100 μL), and the quantity of enzyme thus formed was measured by its amidasic activity on the synthetic substrate CBS 42.46 (200 μL). Calibration curve was obtained with a linearity range 3−150% (% C = 546.5365 DO/min − 3.1556; r^2 = 1.000) with STA Unicalibrator (608035). Normal plasma (STA PreciClot Plus I, Diagnostica Stago, Roche, Portugal), and pathological plasma (STA PreciClot Plus II, Diagnostica Stago, Roche, Portugal) were used for checking the accuracy and reproducibility of results.

Prothrombin Factor Levels. After the addition of compound solution or water (50 μL) citrated pooled human plasma (100 μL) was diluted with factor diluent solution (350 μL, IZASA, Portugal). The clotting time (PT) was determined after mixing the plasma with factor II deficient plasma. High and low calibration curves were prepared with calibrated plasma using dilutions automatically performed by the instrument (ACL100 coagulometer, IZASA, Portugal). Linearity range was 1.56−6.25% (r^2 = 0.999) for low calibration curve, and linearity range was 25−100% (r^2 = 0.999) for high calibration curve. Normal plasma (STA PreciClot Plus I, Diagnostica Stago, Roche, Portugal) and pathological plasma (STA PreciClot Plus II, Diagnostica Stago, Roche, Portugal) were used for checking the accuracy and reproducibility of results.

Thromboelastography. Blood samples from healthy donors were collected into siliconized Vacutainer tubes containing 3.8% trisodium citrate such that a ratio of citrate/whole blood of 1:9 (v/v) was maintained. Sulfated compounds were dissolved in saline and diluted in the whole blood (1:16) to a final concentration of $(3.12-6.25) \times 10^{-4}$ M. In the control group saline was added to the whole blood (1:16). The TEG assay was performed as previously described[13] using a thromboelastograph coagulation analyzer Rotem delta (Pentapharm GmbH, Germany). All disposable supplies were purchased from Pentapharm GmbH.

In Vivo Studies. 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) and as previously described.[13]

Platelet Function Closure Times. Blood samples from healthy donors were collected into tubes containing hirudin at a final concentration of 25 μg/mL and analyzed within 0.5−1 h after collection. In vitro platelet function was evaluated using a PFA-100 device (Dade Behring, Frankfurt, Germany) as previously reported.[13]

Multiplate Electrical Impedance Aggregometry. Blood samples from healthy donors were collected into tubes containing hirudin at a final concentration of 25 μg/mL and analyzed within 0.5−1 h after collection. Stock solutions of compounds **7**, **12**, **13**, ASA, and sodium sulfate were prepared in saline and nonsulfated parent compound **5** in DMSO (the final concentration of DMSO was 0.5% to eliminate the effect of the solvent on the aggregation). Tested solutions (125 μL, saline) were added to whole blood (1875 μL) to a final concentration of 6.25×10^{-4} M. The assays were carried out according to the respective instructions of the manufacturer (multiplate, Dynabyte, Munich, Germany): An amount of 300 μL of whole blood at room temperature was added to 300 μL of preheated saline at 37 °C. After an incubation time of 180 s, 20 μL of the selected agonist solution (Dynabyte, Munich, Germany) was added, giving final concentrations of thrombin receptor-activating peptide (TRAP-6) at 32 μM (TRAP test), arachidonic acid at 0.5 mM (ASPI test), collagen at 3.2 μg/mL (Col test), ADP at 6.5 μM (ADP test), or prostaglandin E1 at 9.4 nM (ADP-HS test). The instrument continuously measures the change in resistance during 6 min, which is proportional to the amount of platelets adhering to the electrodes and transforms it to arbitrary "aggregation units" (AU). These are plotted against time (min) and give the area under the curve (AUC) in U (1 U = 10 AU × min), calculated from the mean values of the two curves. The analysis was accepted when the difference between the two curves is <20%.

Statistics. The statistical significance of the difference between control and treated samples was calculated by the nonparametric two-tailed Mann−Whitney test using GraphPad Prism 5 software. $P < 0.05$ was considered significant.

■ ASSOCIATED CONTENT

Supporting Information. Structural characterization and FXa dose−response progress curves. This material is available free of charge via the Internet at http://pubs.acs.org.

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ABBREVIATIONS USED

ATIII, antithrombin III; FXa, factor Xa; PFA, platelet function analyzer; MW, microwave; APTT, activated partial thromboplastin time; PT, prothrombin time; TT, thrombin time; LMWH, low molecular weight heparins; APC, activated protein C; TEG, thromboelastography; ip, intraperitoneal; GOT, glutamic−oxaloacetic transaminase; GPT, glutamate−pyruvate−transaminase; Col, collagen; TRAP, thrombin receptor-activating peptide; ADP, adenosine 5′-diphosphate; EPI, epinephrine; ASA, acetylsalicylic acid

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