Pergamon

SCIENCE DIRECT®

Bioorganic & Medicinal Chemistry Letters 13 (2003) 679–683

BIOORGANIC &
MEDICINAL
CHEMISTRY
LETTERS

Exploring New Non-sugar Sulfated Molecules as Activators of Antithrombin

Gunnar T. Gunnarsson and Umesh R. Desai*

Department of Medicinal Chemistry, Virginia Commonwealth University,
410 N. 12th Street, PO Box 980540, Richmond, VA 23298, USA

Received 3 September 2002; accepted 14 November 2002

Abstract—New non-sugar, small, sulfated molecules, based on our de novo rationally designed activator (−)-epicatechin sulfate (ECS), were investigated to bind and activate antithrombin, an inhibitor of plasma coagulation enzyme factor Xa. For the activators studied, the equilibrium dissociation constant (K_D) of the interaction with plasma antithrombin varies nearly 53-fold, with the highest affinity of 1.8 μM observed for morin sulfate, while the acceleration in factor Xa inhibition varies 2.6-fold. The results demonstrate that antithrombin binding and activation is a common property of these small sulfated molecules and suggests plausible directions for designing more potent activators.

Antithrombin, a plasma glycoprotein, is a major regulator of the blood coagulation cascade. It performs this function in the presence of heparin by efficiently inhibiting several pivotal proteinases present in the blood, especially factor Xa.[1,2] Heparin, a naturally occurring, highly sulfated, linear polysaccharide ($M_R \sim 14$ kDa), accelerates the antithrombin inhibition of factor Xa several hundred-fold.[3] This forms the basis for heparin-based anticoagulant therapy, which has reached annual cost of over $3 billion for the drug alone.[4] Yet, the anticoagulant therapy is beset with many undesirable effects including bleeding complications, risk of hemorrhage, osteoporosis and inconsistent patient response.[4–7]

This situation is improved with low molecular weight heparins that show reduced bleeding complications.[8,9] Recently, a sequence-specific five-residue saccharide has been introduced in the market. Synthetic pentasaccharide Arixtra,™ based on DEFGH# structure, has been shown to possess much reduced risk of hemorrhage,[10,11] confirming that the risk of heparinopathy arises primarily from large fragments of highly anionic chains.[12–14]

Detailed structure–activity studies have shown that a specific three-residue sequence, called the DEF sequence (Fig. 1), is critical for enhancement in the rate of factor Xa inhibition.[15–18] The binding of trisaccharide DEF induces conformational movements in the inhibitor, especially in an exposed 15-residue reactive center loop (RCL) sequence that contains the scissile bond.[19–21] DEF forms part of the five-residue sequence DEFGH that binds in an electropositive domain in antithrombin, called the pentasaccharide–binding site, which is located nearly 20 Å away from the RCL.[19] The interaction of the carboxylate and sulfate moieties (Fig. 1) with the electropositive side chains of antithrombin results in the transmission of binding energy to the RCL. This allosteric phenomenon is called the conformational activation of antithrombin and is critical for the accelerated inhibition of factor Xa.[19]

We reasoned that high–affinity, small, synthetic, non-sugar molecules could be designed utilizing the extensive structural and mechanistic information available on heparin–antithrombin interaction.[17–21] Using hydropathic interaction (HINT) analysis, we designed (−)-epicatechin sulfate (ECS, Fig. 1), a non-saccharide sulfated flavan-3-ol molecule, to activate antithrombin.[22] ECS was found to bind antithrombin with an affinity comparable to DEF, as expected. More importantly, ECS accelerated the antithrombin inhibition of factor Xa nearly 10-fold, the first small non-sugar organic mole-

---

*Corresponding author. Tel.: + 1-804-828-8486; fax: + 1-804-828-7625; e-mail: urdesai@vcu.edu

Abbreviations: DEFGH, sequence-specific pentasaccharide of heparin (see ref 17 or 22 for structure); DEF, trisaccharide DEF of the pentasaccharide DEFGH; RCL, reactive center loop; HINT, hydropathic interaction analyses.

0960-894X/03/$ - see front matter © 2003 Elsevier Science Ltd. All rights reserved.
doi:10.1016/S0960-894X(02)01055-7

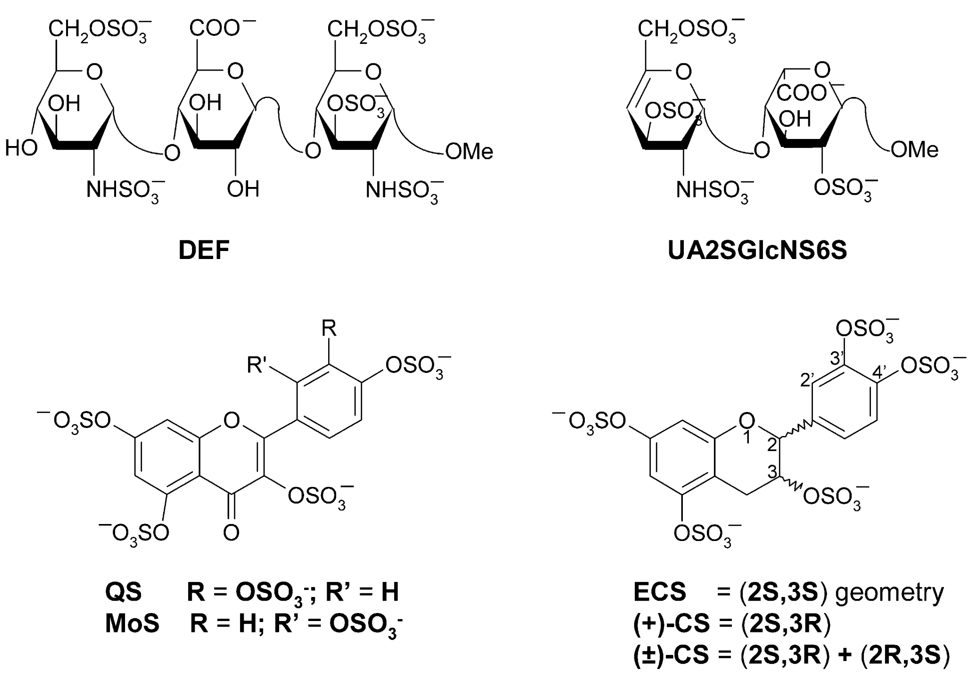


Figure 1. Structures of sulfated molecules and saccharide activators. DEF represents the specific trisaccharide sequence in heparin, which formed the basis for the rational design of activator ECS.[22] Disaccharide UA2SGlcNS6S is approximately equivalent to the sulfated flavonoids in molecular size and was used as a reference. Generic positions 1, 2, 3 are marked in the

cule known to activate the inhibitor. However, the antithrombin activation achieved with ECS is small in comparison to nearly 300-fold observed,[22] and targeted for, with DEF.

ECS is a small scaffold dramatically different from the known saccharide-based activators. The bicyclic–unicyclic scaffold is synthetically more accessible than the heparin-based activators.[23,24] Moreover, the organic scaffold of ECS may offer other potential advantages, such as greater non-ionic binding energy for oral bioavailability, in comparison to the heparin (or saccharide) activators.

Results and Discussion

To discover better activator(s) in the bicyclic–unicyclic class of compounds, we studied the flavan and flavone series of molecules (Fig. 1). Each potential activator contains five sulfate moieties at differing positions introducing considerable configurational differences. Further, a comparison of the ground state conformational preferences of these molecules indicate that the unicyclic ring may orient from an 'east' to a 'south' position, a change in orientation of nearly 90° (Fig. 2). Thus, as a group these bicyclic–unicyclic molecules represent considerable structural diversity. A disaccharide UA2SGlcNS6S containing five negatively charged groups, and with MW in the range of the sulfated flavonoids, was also studied for comparative purposes.

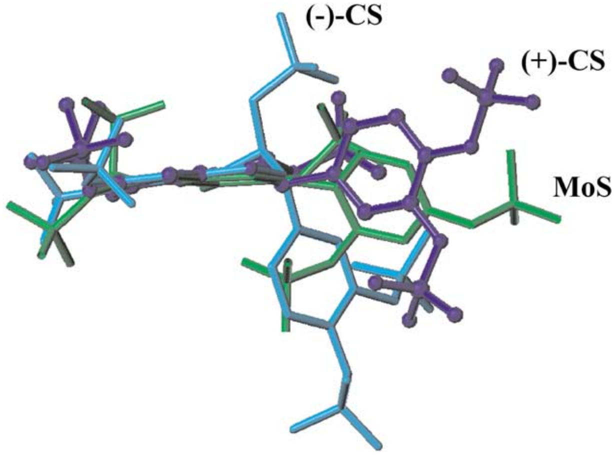
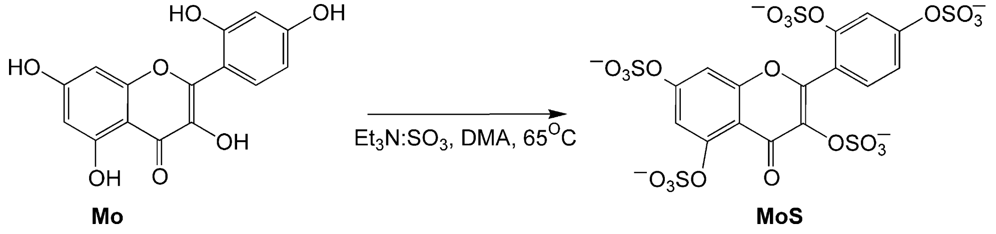


Figure 2. Comparison of the conformational differences in sulfated flavonoid activators. Sybyl 6.7 (Tripos Associates) rendering of (−)-CS (capped stick format in cyan), (+)-CS (ball and stick format in blue) and MoS (flat stick format in green) structure. The bicyclic ring of each activator was superimposed to bring out the differences in the conformational preferences. The unicyclic ring changes its orientation from 'east' [(+)-CS] to 'south' [(−)-CS], a difference of ~90°. The remaining molecules studied showed structures within the boundaries defined by these two.

Sulfation of parent flavonoids was achieved with triethylamine–sulfur trioxide complex [Scheme 1, representative example (morin) is shown].[25] The conformational activation of antithrombin was evaluated at pH 6.0, I 0.025, 25 °C[26] from the enhancement in the second-order rate constant for the inhibition of factor Xa in the presence of activator (k_{ACT}) in comparison to its absence (k_{UNCAT}).[27] The profiles of factor Xa inhibition in the presence of each activator are shown in Figure 3. The profiles indicate that all sulfated flavonoids and reference disaccharide increase the rate of antithrombin inhibition of factor Xa. However, considerable differences are apparent. Analysis of the data using equation I[27] suggests that the second-order rate constants (k_{ACT}) range from 2770 to 7100 M^{-1} s^{-1}, a range of nearly 2.6-fold (Table 1). Alternatively, sul-



Scheme 1.

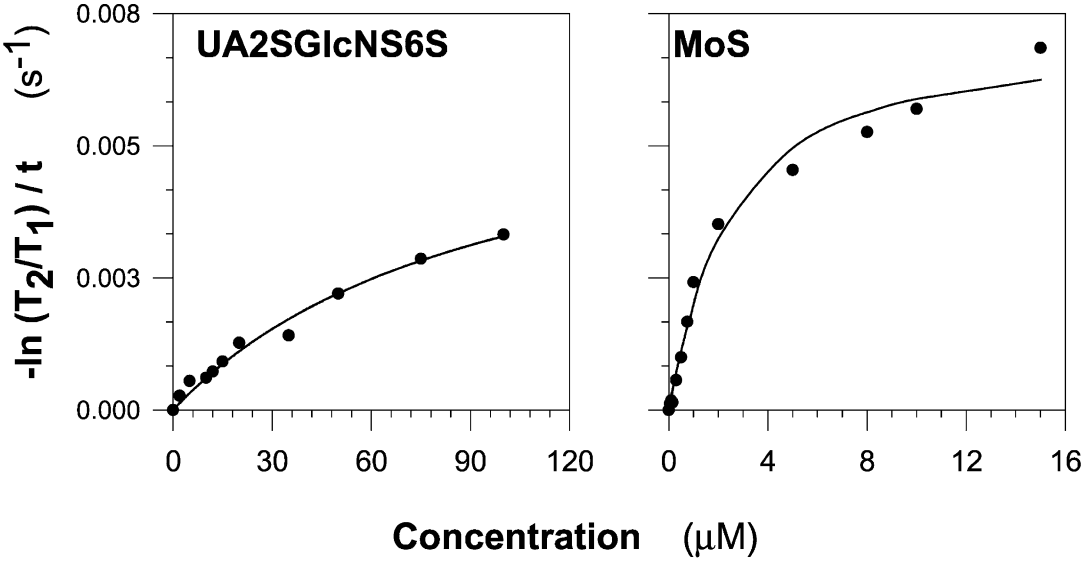


Figure 3. Representative profiles of acceleration in antithrombin inhibition of factor Xa achieved with sulfated molecules. The profile for the least and the most active activator is shown. The inhibition of factor Xa in the presence of increasing concentrations of the activator was monitored from the decrease in the concentration of residual active enzyme under pseudo-first-order conditions at pH 6.0, I 0.025, 25 °C.[27] Note the significantly different concentration at which saturation is achieved for the two. See text for details.

Table 1. Equilibrium dissociation constant (K_D) and second-order rate constant of factor Xa inhibition (k_{ACT}) for interaction of sulfated flavonoids with plasma antithrombin[a]

	K_D (µM)	k_{ACT} (M^{-1} s^{-1})	Acceleration k_{ACT}/k_{UNCAT}
DEF[b]	2.0±0.6	45,000±2000	320±25[c]
UA2SGlcNS6S	95±22	6400±900	19.7±4.3
QS	17.0±3.9	5700±600	17.5±3.2
MoS	1.8±0.4	7100±400	21.8±2.9
(−)-ECS	10.7±1.1	3380±215	10.4±1.5
(+)-CS	3.5±0.5	6750±475	20.8±3.1
(±)-CS	26.1±1.6	2770±170	8.5±1.2

[a] The K_D and k_{ACT} were simultaneously determined from the non-linear regressional analysis of the decrease in the residual concentration of active factor Xa following incubation at 25 °C in pH 6.0, I 0.025 buffer with plasma antithrombin in the presence of varying concentrations of the sulfated activator.[27] A k_{UNCAT} value of 325±25 $M^{-1}s^{-1}$ for antithrombin inhibition of factor Xa was determined independently under identical conditions.[31,32] Errors shown represent ± 1 SE.

[b] Taken from ref 17.

[c] k_{UNCAT} was 140±10 M^{-1} s^{-1} in this experiment.

fated flavanoids accelerate the antithrombin inhibition of factor Xa 8.5±1.2-fold to 21.8±2.9-fold (Table 1).

The equilibrium dissociation constants (K_D) of antithrombin–activator interaction, obtained from the non-linear regressional analysis of the factor Xa inhibition data[27] (Fig. 3), ranged from 1.8 to 26 µM for the sulfated flavonoids, while it was 95 µM for the disaccharide UA2SGlcNS6S. Thus the binding affinities span a range of 52.8-fold. It is interesting to note that this study has uncovered morin sulfate (MoS), a molecule that binds antithrombin nearly 5-fold tighter than our first rationally designed activator ECS (~11 µM).[22]

The observation that nearly all sulfated flavonoids activate antithrombin for accelerated inhibition of factor Xa is interesting because, unlike heparin (or its derivatives), the flavonoids are not sugar based. Further, a recent report indicated that some sulfated flavone derivatives, containing a 5-hydroxyl group or a 3-acetyl group (or lacking sulfate groups at these positions) in the bicyclic ring (see Fig. 1 for numbering), did not accelerate factor Xa inhibition at concentrations as high as 1 mM.[28]

The similar acceleration achieved with different sulfated flavonoids at saturation suggests that these activators most likely utilize similar binding and activation mechanism. Although the number of molecules investigated here is small, it is likely that the ~20-fold acceleration may represent a maximum for these small activators. This acceleration is considerably lower than that with the sequence-specific trisaccharide[1,17,18] and suggests that the smaller sulfated flavonoids perhaps do not recognize the pentasaccharide–binding site in antithrombin. It is known that full-length heparin, in addition to its high-affinity binding with the pentasaccharide-binding site, interacts with a small region formed by Arg129, Arg132, Lys133 and Lys136 residues in antithrombin that is adjacent to the pentasaccharide-binding site.[29] This region has also been shown to play an important role in the transmission of the heparin binding energy from pentasaccharide-binding site to the RCL.[30]

It is interesting to note that despite the identical number of negative charges on each activator, the binding affinities differ greatly. This suggests considerable specificity in the interaction, especially for MoS. This is the only activator in which a sulfate group is present in an ortho orientation (2′-position) in the unicyclic ring (see Fig. 1). Electronic and/or steric interactions of this sulfate with the 3-OSO_3^- group of the bicyclic ring (Fig. 1) may favor a specific conformation for MoS conducive for tight binding. Thus, the MoS structure represents a lead candidate for further structural manipulations. Natural activator DEF is approximately 12 Å long whereas our sulfated flavanoids span a distance of ~10 Å. Thus, introduction of a one or two-carbon linker between the bicyclic and unicyclic ring may be expected to result in better activators.

To conclude, we have identified several synthetic sulfated flavonoids with measurable antithrombin activation properties. These small synthetic non-sugar molecules represent the first class of activators outside of the traditional heparin or heparin-based anticoagulants. Morin sulfate represents an interesting lead structure for the design of better activators.

Acknowledgements

We thank Profs. Steven Olson and Robert J. Linhardt for the generous gift of human antithrombin and heparin disaccharide UA2SGlcNS6S, respectively. This work was supported by the American Heart Association (U.R.D.) and by a pre-doctoral fellowship from AHA-Mid-Atlantic Association (GTG).

References and Notes

1. Björk, I.; Olson, S. T. *Adv. Exp. Med. Biol.* **1997**, *425*, 17.
2. Gettins, P. G. W.; Patston, P. A.; Olson, S. T. *Serpins: Structure. Function and Biology*; RG Landes and Company: Austin, TX, 1996.
3. Rosenberg, R. D.; Damus, P. S. *J. Biol. Chem.* **1973**, *248*, 6490.
4. Fareed, J.; Hoppensteadt, D. A.; Bick, R. L. *Sem. Thromb. Hemost.* **2000**, *26* (Suppl. 1), 5.
5. Boneu, B. *Thromb. Res.* **2000**, *100*, V113.
6. Cohen, M. *Thromb. Res.* **2000**, *100*, 131.
7. Pineo, G. F.; Hull, R. D. *Annu. Rev. Med.* **1997**, *48*, 79.
8. Turpie, A. G. *Semin. Thromb. Hemost.* **2002**, *28* (Suppl. 3), 3.
9. Mukherjee, D.; Topol, E. J. *Prog. Cardiovasc. Dis.* **2002**, *45*, 139.
10. Eriksson, B. I.; Bauer, K. A.; Lassen, M. R.; Turpie, A. G. G. *New Engl. J. Med.* **2001**, *345*, 1298.
11. Eriksson, B. I.; Bauer, K. A.; Lassen, M. R.; Turpie, A. G. G. *New Engl. J. Med.* **2001**, *345*, 1305.
12. Sinaÿ, P. *Nature* **1999**, *398*, 377.
13. Maccarana, M.; Lindahl, U. *Glycobiology* **1993**, *3*, 271.
14. Petitou, M.; Herault, J. P.; Bernat, A.; Driguez, P. A.; Duchaussoy, P.; Lormeau, J. C.; Herbert, J. M. *Nature* **1999**, *398*, 417.
15. Choay, J.; Lormeau, J.-C.; Petitou, M.; Sinäy, P.; Fareed, J. *Ann. N.Y. Acad. Sci.* **1981**, *370*, 644.
16. Choay, J.; Petitou, M.; Lormeau, J.-C.; Sinäy, P.; Casu, B.; Gatti, G. *Biochem. Biophys. Res. Commun.* **1983**, *116*, 492.
17. Desai, U. R.; Petitou, M.; Björk, I.; Olson, S. T. *J. Biol. Chem.* **1998**, *273*, 7478.
18. Desai, U. R.; Petitou, M.; Björk, I.; Olson, S. T. *Biochemistry* **1998**, *37*, 13033.
19. Huntington, J. A.; Olson, S. T.; Fan, B.; Gettins, P. G. W. *Biochemistry* **1996**, *35*, 8495.
20. Jin, L.; Abrahams, J.-P.; Skinner, R.; Petitou, M.; Pike, R. N.; Carrell, R. W. *Proc. Natl. Acad. Sci. U.S.A.* **1997**, *94*, 14683.
21. Whisstock, J. C.; Pike, R. N.; Jin, L.; Skinner, R.; Pei, X. Y.; Carrell, R. W.; Lesk, A. M. *J. Mol. Biol.* **2000**, *301*, 1287.
22. Gunnarsson, G. T.; Desai, U. R. *J. Med. Chem.* **2002**, *45*, 1233.
23. Petitou, M.; Duchaussoy, P.; Lederman, I.; Choay, J.; Sinäy, P.; Jacquinet, J.-C.; Torri, G. *Carbohydr. Res.* **1986**, *147*, 221.
24. van Boeckel, C. A. A.; Petitou, M. *Angew. Chem., Int. Ed. Engl.* **1993**, *32*, 1671.
25. Sulfated flavonoids were synthesized by sulfation of parent flavonoid (from either Aldrich or Indofine) with triethylaminesulfur trioxide (Aldrich, 4 molar equivalent per hydroxyl group) in DMA at 65 °C. Briefly, following reaction for 3 h, the mixture was poured into acetone under basic conditions (few mL of triethylamine) and left at 4 °C for 24 h. The crude oil formed at the bottom was washed with acetone and ether, and suspended in 30% sodium acetate. The suspension was filtered to remove extraneous solids and then added dropwise in ethanol to precipitate the sodium salt of the sulfated molecule. The concentration of the sulfated activator was determined from its extinction co-efficient (ε_λ) at either 277 nm (ECS and CS) or 253 nm (MoS and QS). The ε_λ used for ECS and CS (RCS) were 4200 and 3890 M^{-1} cm^{-1}, respectively, while that for QS and MoS were 8400 M^{-1} cm^{-1}, respectively. The IR spectra of each sulfated product indicated a strong band in the region 1250–1260 cm^{-1}, characteristic of $-SO_3^-$ stretch vibrations. A comparison of the 300 MHz ^1H NMR spectra of the starting reactant and the sulfated product in each case shows downfield shift of 0.5–0.9 ppm for protons suggesting sulfation of the hydroxyl groups. Elemental analysis of repeatedly precipitated sulfated product indicated the presence of H_2O, EtOH and Na_2SO_4 molecules. **QS**: ^1H NMR (^2H_2O) δ: 7.4 (d, 1H, $^4J = 2.4$ Hz), 7.5 (d, 1H, $^4J = 2.1$ Hz), 7.6 (d, 1H, $^3J = 8.7$ Hz), 8.0 (dd, 1H, $^3J = 8.7$ Hz, $^4J = 2.4$ Hz), 8.1 (d, 1H, $^4J = 2.1$ Hz); Elemental analysis: calcd (%) for $C_{15}H_9O_{21}S_5Na_5 + 9H_2O + 0.5Na_2SO_4$: C, 17.2; H, 2.2; S, 16.9. Found (%) C, 16.5; H 2.15, S, 17.7. **MoS**: ^1H NMR (^2H_2O) δ: 7.35 (dd, 1H, $^3J = 8.4$ Hz, $^4J = 2.4$ Hz), 7.4 (d, 1H, $^3J = 8.4$ Hz), 7.5 (d, 1H, $^4J = 2.4$ Hz), 7.7–8.0 (m, 2H); Elemental analysis: calcd (%) for $C_{15}H_5O_{22}S_5Na_5 + 4H_2O + 1C_2H_5OH$: C, 21.9; H, 2.1; O, 46.4. Found (%) C, 21.0; H 2.2, O, 47.5. **ECS**: ^1H NMR (^2H_2O) δ: 3.10 (dd, 1H, $^2J = 18$ Hz, $^3J = 4.2$ Hz, Ar-C$\underline{H}_2$), 3.45 (dd, 1H, $^2J = 18$ Hz, $^3J = 6$ Hz, Ar-C$\underline{H}_2$), 5.00 (m, 1H, C$\underline{H}$–OSO_3^-), 5.10 (d, 1H, $^3J = 8.1$ Hz, O-C$\underline{H}$-Ar), 6.80-7.70 (m, 5H, Ar); Elemental analysis: calcd (%) for $C_{15}H_9O_{21}S_5Na_5 + 6H_2O + 0.5Na_2SO_4$: C, 17.9; H, 2.1; S,

17.8. Found (%) C, 17.29; H 2.21, S, 18.00. CS: ^{1}H NMR (^2H_2O) δ: CS: ^{1}H NMR (^2H_2O): 3.15 (dd, 1H, $^2J=17.4$ Hz, $^3J=4.8$ Hz, Ar–$\underline{\mathrm{CH}_2}$), 3.6 (dd, 1H, $^2J=17.4$ Hz, $^3J=7.2$ Hz, Ar–$\underline{\mathrm{CH}_2}$), 5.0 (m, 1H, $\underline{\mathrm{CH}}$–OSO_3^-), 5.15 (d, 1H, $^3J=4.5$ Hz, O–$\underline{\mathrm{CH}}$–Ar), 6.90–7.60 (m, 5H, Ar); Elemental analysis: calcd (%) for $C_{15}H_9O_{21}S_5Na_5 + 9H_2O + 0.0Na_2SO_4$: C, 18.7; H, 2.8; S, 16.7. Found (%) C, 18.87; H 2.96, S, 16.36. RCS: ^{1}H NMR (^2H_2O) δ: ^{1}H NMR (^2H_2O): 3.15 (m, 1H, Ar–$\underline{\mathrm{CH}_2}$), 3.6 (m, 1H, Ar–$\underline{\mathrm{CH}_2}$), 5.0 (m, 1H, $\underline{\mathrm{CH}}$–OSO_3^-), 5.20 (m, 1H, O–$\underline{\mathrm{CH}}$–Ar), 6.90-7.50 (m, 5H, Ar); Elemental analysis: calcd (%) for $C_{15}H_9O_{21}S_5Na_5 + 8H_2O + 3.0Na_2SO_4$: C, 13.1; H, 1.9; S, 18.6. Found (%) C, 12.88; H 1.81, S, 17.97.

26. Antithrombin activation studies were performed at 25 °C and in 20 mM sodium phosphate buffer, containing 0.1 mM EDTA and 0.1% (w/v) PEG 8000, adjusted to pH 6.0. The ionic strength (I) of buffer in the absence of any added salt is 0.025.

27. The kinetics of inhibition of factor Xa (Haematologic Technologies, Essex Junction, VT, USA) by human antithrombin (a gift from Professor Steven T. Olson) alone was determined spectrophotometrically following literature procedures.[31,32] The kinetics of the antithrombin inhibition of factor Xa in the presence of sulfated flavonoids was measured under pseudo-first-order conditions. Briefly, a fixed 10 nM concentration of factor Xa was incubated with plasma antithrombin (1 μM) and the sulfated flavonoid (0–200 μM) in pH 6.0 buffer at 25 °C.[19] After incubation for 10 min, the reactions were quenched with 900 μL of 100 μM Spectrozyme FXa in 20 mM sodium phosphate buffer, pH 7.4, containing 100 mM sodium chloride, 0.1 mM EDTA, 0.1% (w/v) PEG 8000. The residual factor Xa activity was then measured spectrophotometrically from the initial rate of substrate hydrolysis at 405 nm. The second-order rate constant of factor Xa inhibition (k_{ACT}) and equilibrium dissociation constant (K_{D}) were determined simultaneously using equation I (deduced from pseudo-first ordered reaction equation for single turnover kinetics in the presence (T_2, $k_{\mathrm{OBS}} = k_{\mathrm{UNCAT}}\,[\mathrm{AT}]_{\mathrm{O}} + k_{\mathrm{ACT}}\,[\mathrm{AT/ACT}]_{\mathrm{O}}$) and absence ($T_1$, $k_{\mathrm{OBS}} = k_{\mathrm{UNCAT}}\,[\mathrm{AT}]_{\mathrm{O}}$) of activator (ACT), where T_{O} is the initial activity of the enzyme and the concentration of the antithrombin–activator complex ($[\mathrm{AT{:}ACT}]_{\mathrm{O}}$) is obtained from the quadratic equilibrium binding equation).

$$\frac{\ln(T_1/T_2)}{t} = k_{\mathrm{ACT}}$$

$$\times \frac{([AT]_{\mathrm{O}} + [ACT]_{\mathrm{O}} + K_{\mathrm{D}}) - \sqrt{([AT]_{\mathrm{O}} + [ACT]_{\mathrm{O}} + K_{\mathrm{D}})^2 - 4[AT]_{\mathrm{O}}[ACT]_{\mathrm{O}}}}{2[AT]_{\mathrm{O}}}$$

28. Guglielmone, H. A.; Agnese, A. M.; Núñez Montoya, S. C.; Cabrera, J. L. *Thromb. Res.* **2002**, *105*, 183.

29. Arocas, V.; Turk, B.; Bock, S. C.; Olson, S. T.; Björk, I. *Biochemistry* **2000**, *39*, 8512.

30. Meagher, J.; Olson, S.; Gettins, P. *J. Biol. Chem.* **2000**, *275*, 2698.

31. Olson, S. T.; Björk, I.; Shore, J. D. *Methods Enzymol.* **1993**, *222*, 525.

32. Olson, S. T.; Björk, I.; Sheffer, R.; Craig, P. A.; Shore, J. D.; Choay, J. *J. Biol. Chem.* **1992**, *267*, 12528.