

## Supporting Information

# Flavonoids with an oligo-polysulfated moiety: a new class of anticoagulant agents

*Marta Correia-da-Silva<sup>§</sup>, Emília Sousa<sup>§</sup>, Bárbara Duarte<sup>‡</sup>, Franklim Marques<sup>‡</sup>, Félix Carvalho<sup>¶</sup>, Luís M. Cunha-Ribeiro<sup>#</sup>, and Madalena M. M. Pinto<sup>§\*</sup>*

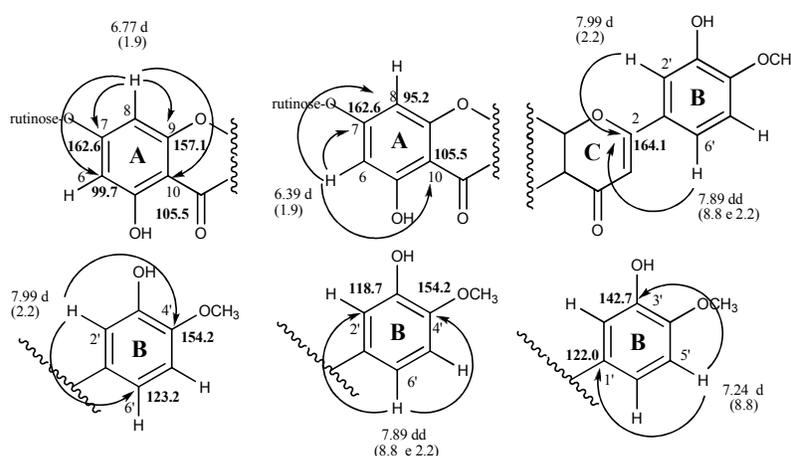
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## Structural characterization.

The IR spectra of all the sulfated derivatives (**7-13**) showed the presence of two strong bands corresponding to the S=O (ca 1261 cm<sup>-1</sup>) and C-O-S (ca 1047 cm<sup>-1</sup>) groups and a band at ca. 806 cm<sup>-1</sup> corresponding to the S-O group. The presence of a band at 3600-3400 cm<sup>-1</sup> characteristic for OH group indicated that the sulfation did not occur for all hydroxyl groups in compounds **7-12**. The MS allowed establishing the number of sulfate groups introduced: six for compounds **7** and **8**, nine for compounds **9** and **10**, two for compounds **11** and **12** and ten for compound **13**. The <sup>1</sup>H NMR and <sup>13</sup>C NMR data of sulfated compounds (**7-13**) allowed the unambiguous establishment of the positions of sulfate groups.

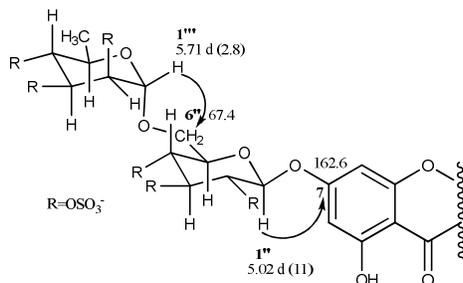
The multiplicity and coupling constants for the protons observed in the <sup>1</sup>H NMR spectra for compounds **7-12** showed in common the signals of one proton bonded hydroxyl group at  $\delta_H$  11.97–12.83 ppm and four aromatic protons corresponding to the phenyl rings ( $\delta_H$  6-8 ppm). The <sup>13</sup>C NMR spectra showed besides the carbon signals of the aromatic rings ( $\delta_C$  112-164 ppm) the carbonyl carbon of flavonoid scaffold ( $\delta_C$  172-197 ppm). The chemical shifts of the carbon atoms not directly bonded to proton atoms were inferred from HMBC correlations as exemplified for compound **7** in Figure S1.



**Figure S1.** Main connectivities found in the HMBC of the flavonic moiety of sulfated diosmin (**7**).

When the proton chemical shifts for the sulfated flavonoids **11** and **12** were compared with those of the respective building blocks **5** and **6**, a downfield shift for H-2', H-6, and H-8 signals were observed and the 7-OH and 3'-OH signals were only present in the building blocks **5** and **6**. These alterations in the <sup>1</sup>H NMR spectra indicated that the sulfate groups were located at positions 3' and 7 in the aglycone. When the proton chemical shifts for the sulfated rutin derivatives **9** and **13** were compared with those of the respective building block **3**, a downfield shift for H-2', H-5', H-6 and H-8 proton resonances were verified and 7-OH, 3'-OH and 4'-OH signals were only present in compound **3**. Similarly, the presence of a 5-sulfate in derivative **13** was established when comparing to derivative **9** since the 5-OH signal was only present in compound **9**.

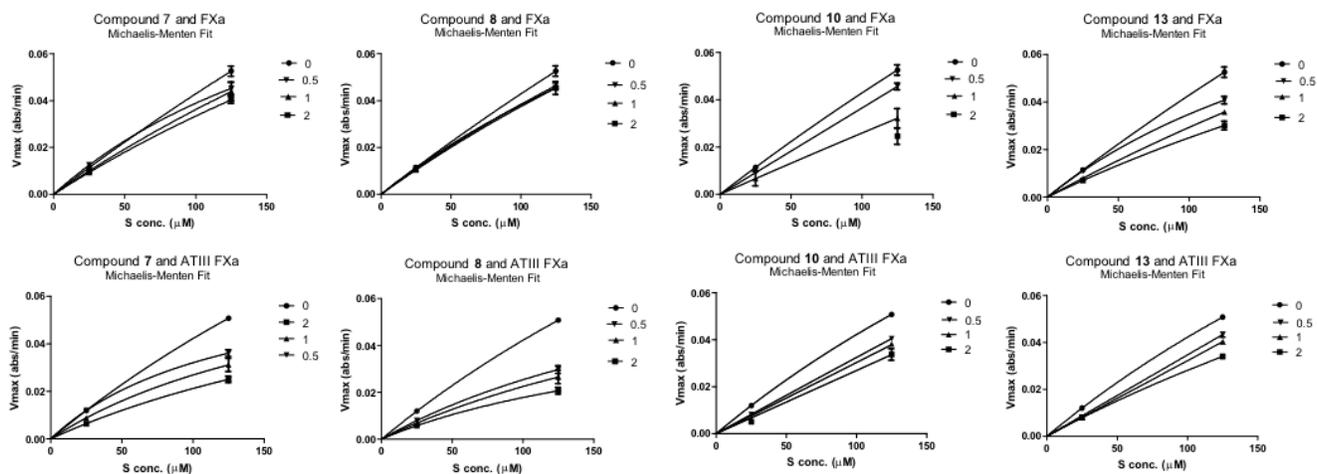
The presence of  $\beta$ -D-glucopyranose and  $\beta$ -L-rhamnopyranose units were confirmed by the presence of two anomeric protons and a methyl signal at  $\delta_H$  0.93-1.10 ppm in the <sup>1</sup>H NMR spectra and by the presence of two anomeric carbon signals at  $\delta_C$  102 ppm, nine sugar carbons at  $\delta_C$  62.0-76.2 ppm and a methyl group at  $\delta_C$  14.8-18.0 ppm in the <sup>13</sup>C NMR spectra. HMBC correlations between H-1'' and C-6'' indicated that rhamnose was linked to the C-6'' of the glucose, confirming the presence of the rutinose unit [rhamnosyl(1-6)glucoside] as a sugar moiety. The attachment of the rutinose to the aglycone was confirmed from the correlations observed in the HMBC spectra between H-1'' and C-7 for flavonoids 7-O-rutinosides (**7** and **8**), as exemplified for compound **7** (Figure S2), and between H-1'' and C-3 for flavonoids 3-O-rutinosides (**9**, **10**, and **13**).



**Figure S2.** Main connectivities found in the HMBC of the rutinose moiety of sulfated diosmin (**7**).

When the proton chemical shifts from the  $^1\text{H}$  NMR spectra of sulfated flavonoids glycosides (**7-10** and **13**) were compared with those from the  $^1\text{H}$  NMR spectra of the building blocks **1-4**, respectively, downfield shifts of the sugar proton resonances were observed and the OH resonances between  $\delta_{\text{H}}$  4.5-5.5 ppm were only observed for compounds **1-4**. Furthermore, upfield shifts of the anomeric carbons (C-1'', C-1''') signals were observed for all the sulfated flavonoids glycosides (**7-10** and **13**) in the  $^{13}\text{C}$  NMR spectra. These alterations to the NMR spectra indicated that all sugar hydroxyls were sulfated.

### Michaelis-Menten fit of sulfated flavonosides **7**, **8**, **10**, and **13** against FXa and ATIII/FXa.



**Figure S3.** Sulfated flavonosides **7**, **8**, **10**, and **13** against FXa and ATIII/FXa fitted by the Michaelis-Menten equation (non linear regression analysis).

### Experimental Section.

**Diosmetin (5).** Diosmin (**1**, 2 g, 3.29 mmol) in 200 mL of a mixture of sulfuric acid-acetic acid-water (1:30:19) was kept under reflux for 12 h. After cooling, the insoluble precipitate was filtered and washed with water afforded diosmetin (**5**, 0.869 g, 2.894 mmol). Yellow solid, yield 88%; mp 250-252 °C, water (253-254 °C, ethanol/ethyl acetate). IR (KBr) $_{\text{vmax}}$ : 3500-3200, 2925, 1658, 1612, 1506, 1437, 1259.  $^1\text{H}$  NMR (DMSO- $d_6$ , 300.13 MHz)  $\delta$ : 7.55 (1H, dd,  $J$  = 8.5 and 2.3 Hz, H-6'), 7.49 (1H, d,  $J$  = 2.3 Hz, H-2'), 7.10 (1H, d,  $J$  = 8.5 Hz, H-5'), 6.76 (1H, s, H-3), 6.48 (1H, d,  $J$  = 2.0 Hz, H-8), 6.20 (1H, d,  $J$  = 2.0 Hz, H-6), 3.87 (3H, s, 4'-OCH<sub>3</sub>).  $^{13}\text{C}$  NMR (DMSO- $d_6$ , 75.47 MHz)  $\delta$ : 181.8 (C-4), 164.2 (C-7), 163.6

(C-2), 161.5 (C-5), 157.4 (C-9), 151.2 (C-4'), 146.8 (C-3'), 123.0 (C-6'), 118.8 (C-1'), 113.0 (C-2'), 112.2 (C-5'), 103.8 (C-10), 103.6 (C-3), 98.9 (C-6), 94.0 (C-8), 55.8 (OCH<sub>3</sub>). MS (EI): 300 ([M]<sup>+</sup>).

**Hesperetin (6).** Hesperidin (**2**, 3 g, 4.91 mmol) in 200 mL of a mixture of sulfuric acid-acetic acid-water (1:30:19) was kept under reflux for 12 h. The reaction mixture was cooled and extracted with CH<sub>2</sub>Cl<sub>2</sub> (30 mL) and AcOEt (3×30 mL). The organic layers were collected, washed with water, dried with anhydrous sodium sulfate and the solvent was removed by evaporation under reduced pressure, afforded hesperetin (**6**, 1.46 g, 4.827 mmol). Brown solid, yield 98%; mp 220-222°C, ethanol (226-228°C, CH<sub>2</sub>Cl<sub>2</sub>/AcOEt). IR (KBr)<sub>vmax</sub>: 3500-3300, 2954, 1637, 1579, 1471, 1263. <sup>1</sup>H NMR (DMSO-*d*<sub>6</sub>, 300.13 MHz) δ: 12.15 (1H, s, 5-OH), 10.84 (1H, s, 7-OH), 9.14 (1H, brs, 3'-OH), 6.95-6.86 (3H, m, H-2', H-5', H-6'), 5.90-5.88 (2H, m, H-8 e H-6), 5.44 (1H, dd, *J* = 12.3 and 3.0 Hz, H-2), 3.78 (3H, s, 4'-OCH<sub>3</sub>), 3.29-3.16 (1H, m, H-3 *trans*), 2.71 (1H, dd, *J* = 17.1 and 3.0 Hz, H-3 *cis*). <sup>13</sup>C NMR (DMSO-*d*<sub>6</sub>, 75.47 MHz) δ: 196.3 (C-4), 166.7 (C-7), 163.5 (C-5), 162.9 (C-9), 147.9 (C-4'), 146.5 (C-3'), 131.2 (C-1'), 117.8 (C-6'), 114.1 (C-2'), 112.0 (C-5'), 101.8 (C-10), 95.8 (C-6), 95.0 (C-8), 78.3 (C-2), 55.7 (OCH<sub>3</sub>), 42.1 (C-3). MS (EI): 302 ([M]<sup>+</sup>).

**Rutin 2'',2''',3',3'',3''',4',4'',4''',7-O-nonasulfate (9).** The precipitate obtained in procedure **b** (see Experimental Section) was further purified from other salts (monitored by IR analysis) using a Spectra/Por 6 Regenerated Cellulose MWCO1,000.

**3'',4''-Bis(2-O-sulfate ethoxy)-7-(2-O-sulfate ethoxy)-rutin (10).** The precipitate obtained in procedure **b** (see Experimental Section) was further purified from other salts (monitored by IR analysis) using a Spectra/Por 6 Regenerated Cellulose MWCO1,000.

**Diosmetin 3',7-O-disulfate (11).** The precipitate obtained in procedure **b** (see Experimental Section) corresponded to an inorganic salt (monitored by IR analysis). Treatment of the filtrate by removing the solvent under reduced pressure afforded a crude oil. Aqueous solution of 30% of tetrabutylammonium (15 mL) was added to the crude oil to afford tetrabutylammonium salts. An inorganic white solid coprecipitated with brown oil. The solid was then separated from the oil by dissolution in water followed by centrifugation. The oil was separated and suspended in 30% aqueous sodium acetate solution to precipitate the sodium salt of the sulfated diosmetin (**11**).

**Hesperetin 3',7-O-disulfate (12).** The precipitate obtained in procedure **b** (see Experimental Section) was further purified by gel chromatography (Sephadex G-10, water) affording the sodium salt of compound **12**.