Supplementary Information to Zhang, Till et al. “Structure-activity relationship of the pro- and anticoagulant effects of Fucus vesiculosus fucoidan” (Thromb Haemost 2014; 111.3)

Experiments

*Phenol-sulfuric acid assay*

The phenol-sulfuric acid assay was used to quantify fucoidan during fractionations. To 300 μL sample or fucose standard, 100 μL 5% (w/v) aqueous phenol was added in a glass tube followed by 1 mL of concentrated sulfuric acid. The reactions were incubated at 100 °C for 10 min. After the reacted solutions were cooled to room temperature, they were transferred to a 96 well plate (200 μL in each cell) and absorbance was measured at 490 nm with a plate reader. Chromatograms were generated by plotting the absorbance as function of retention time or tube number.

*Over- and desulfation of fucoidan*

Over- and desulfation reactions were carried out with a medium sized fucoidan (~50 kD) to allow for size increase through additional sulfation as well as size reduction because of possible depolymerisation during desulfation.

*Oversulfation. (1)*

Fucoidan (0.2 g) was stirred in 4 mL of DMF and 6 mL of pyridine at 90 °C for 30 min under argon. Either 1.4 or 0.4 g of sulfation reagent (sulfur trioxide-pyridine complex) was added to produce fucoidans with two levels of oversulfation. For each preparation, the mixture was stirred at 90 °C for 2 h, cooled to room temperature, and the solvent was decanted. The solid was dissolved in 10 mL of saturated NaHCO₃, solvent was evaporated, the solid was dissolved in 20 mL of DI water, and solvent was evaporated to give a white solid. The product was purified over a Sephadex LH20 gel column followed by a "H-exchange column and then lyophilized.
Desulfation.

Fucoidan was desulfated based on a method by Miller and Blunt (2). A mixture of DMSO (45 mL) and toluene (15 mL) was refluxed under Dean Stark conditions until ~15 mL of solution was removed. The solution was cooled to 120 °C and 1.0 g of fucoidan added. After 5 min, 2.5 mL of pyridine, 0.75 g of pyromellitic acid and 0.5 g of NaF were added. Then, another 5 mL of pyridine was added. The reaction was carried out at 120 °C under argon. To obtain different degrees of desulfation, two aliquots were removed from the mixture at 1 and 2 h. After they were cooled to room temperature, 40 mL of EtOAc was added to each aliquot, stirred for 10 min, and then kept at 5 – 8 °C for 2 h. Each aliquot was filtered and the resulting solid was washed with EtOAc (5 mL). The solid was dissolved in DI water (10-15 mL) and 1.5 mL of saturated NaHCO₃ was added. Evaporation gave a white solid. The solid was again dissolved in 10-15 mL DI water, and the solvent was evaporated. The product was purified over a Sephadex LH20 gel column followed by a ¹H-exchange column and then lyophilized.

Nuclear magnetic resonance (NMR) spectroscopy experiments

A 600 MHz (¹H frequency) Bruker Avance III NMR spectrometer with a dual ¹H/¹³C-Cryoprobe was used to analyze the fucoidan starting material and the fractions. Data for the over- and desulfated fucoidans were acquired on either the 600 MHz NMR spectrometer or an 850 MHz Bruker Avance III NMR spectrometer with a ¹H/¹³C/¹⁵N/³¹P cryoprobe. Samples were dissolved in ~0.6 mL D₂O containing 0.02% (w/v) sodium 2,2,3,3-d₄-(trimethylsilyl)propionate (TMSP, 2,2,3,3-D₄, 98%). Qualitative NMR experiments were used to characterize their structures. One-dimensional (1D) ¹H and 1D ¹³C; 2D phase-sensitive multiplicity-edited Heteronuclear Single Quantum Correlation (HSQC), magnitude-mode Heteronuclear Multiple Bond Correlation (HMBC), and Correlation SpectroscopY (COSY); and 3D HSQC-TOtal Correlation SpectroscopY (TOCSY) NMR spectra were acquired.

Average Mw determination using size-exclusion chromatography and Multi-Angle Laser Light Scattering (SEC-MALLS)

An Agilent HPLC System coupled with Wyatt Technology DAWN HELEOS, QELS (Quasi-Elastic Light Scattering) and Optilab rEX differential refractive index (dRI) detectors was used to measure the Mw and polydispersities of different fucoidans. Each fucoidan (~ 5 mg of solid) was
dissolved in 250 µL of PBS mobile phase, and 50 µL injected on a column, Superdex 200 (10 mm/300 mm, GE Healthcare, USA). The change in refractive index/change in concentration (dn/dc) value (0.113 mL/g) was determined for one F.v. fucoidan and this value used to calculate the Mw of all samples.

**Monosaccharide analysis by high-performance anion-exchange chromatography (HPAEC) (3)**

Each sample was dissolved in 2 M trifluoroacetic acid (TFA). Hydrolysis was carried out at 100 °C for 5 h to obtain the monosaccharides. Excess TFA was removed, and the hydrolysates dried using a SpeedVac concentrator and then re-dissolved in the same volume of water and pH adjusted to neutral with diluted NaOH.

A Dionex HPAEC (ICS 3000, Dionex) with dual pumps and pulsed amperometric detector (PAD) was used in this study. The Carbopac guard column (4 X 50 mm, Dionex, USA) and Carbopac PA1 analytical column (4 X 250 mm, Dionex, USA) were maintained at 30 °C. The flow rate was set at 1 mL/min. An isocratic 15 mM NaOH (40 min) elution was used. The waveform of the PAD was the Dionex default program for carbohydrates. The injection volume was set to 25 µL. The ion chromatography system was controlled and the data analyzed by Chromeleon software (version 6.80).

**Calibrated automated thrombography (CAT)**

The procoagulant activity of fucoidans was monitored by CAT as described by Hemker et al. (4). As a model for antibody mediated FVIII deficiency, a normal human plasma pool (George King Biomedical, USA) was incubated with anti-human FVIII plasma raised in goat (Baxter Innovations GmbH, Austria). An optimized heat inactivation process ensured that goat coagulation factors in the inhibitor plasma were essentially inactive. The final inhibitor concentration in the plasma was 50 Bethesda units (BU)/mL, a titer we experimentally demonstrated to completely block FVIII activity (5). For specific inhibition of factor XIIa, the plasma was mixed with corn trypsin inhibitor (CTI) (Hematologic Technologies, Inc., USA), providing a final CTI concentration of 40 µg/mL. To each well of a 96 well micro-plate (Immulon 2HB, U-bottom; Thermo Electron), 80 µL of pre-warmed (37 °C) plasma was added. Concentrations of 0-5 µM of each fucoidan preparation were added to the plasma (10 µL). Thrombin generation was monitored at 37 °C in a Fluoroskan Ascent reader (Thermo Labsystems, Finland) using FluCa and PPP-reagents (Thrombinscope BV, The Netherlands).
Thrombin generation was triggered by 1 pM tissue factor (TF) and 4 µM phospholipids and started by dispensing 20 µL of FluCa reagent (Thrombinscope BV, The Netherlands) containing fluorogenic substrate and HEPES buffered CaCl₂ solution (100 mM) into each well. Fluorescence was measured in a Fluoroskan Ascent® reader (Thermo Labsystems, Finland; filters 390 nm excitation and 460 nm emission) at 37 °C. All samples were analyzed in duplicate and in at least two independent assays.

Parameters of the resulting thrombin generation curves were calculated using Thrombinscope™ software (Thrombinscope BV, The Netherlands). Molar thrombin concentrations in the test wells were derived using the thrombin calibrator. Thrombin concentrations at the peak of each curve (peak thrombin, nM), lag time (interval between starting measurement and start of thrombin generation), peak time (interval between starting measurement and peak thrombin), and endogenous thrombin potential (area under the curve of thrombin concentration versus time) were recorded. To reduce inter-assay variance, thrombin concentrations were expressed as a percentage (%) of normal plasma with normal plasma being 100% and FVIII-inhibited plasma 0%. The procoagulant effect was assessed by plotting peak thrombin against the concentration within the inclining part of the profile. The EC₅₀ value was calculated by data point fitting using a sigmoidal dose-response algorithm (SigmaPlot 12 software).

To evaluate the contact activation of fucoidans, CAT assays were performed in normal human plasma pool as described above with and without CTI.

**TFPI-Dilute Prothrombin Time Assay**

A dPT assay with added TFPI (TFPI-dPT) was used to demonstrate the TFPI-inhibiting effect of *F. v.* fucoidan fractions in normal human plasma (George King Biomedical, USA). Plasma samples were pre-incubated with 0.5 µg/mL full-length TFPI (aa 1-276, constitutively produced by SKHep1 cells and purified to homogeneity) (6) and *F. v.* fractions (0-1 µM) for 15 min at RT. TF reagent TriniClot PT Excel S (Trinity Biotech, Ireland), diluted in Heps-buffered saline 1:200 with 0.5 % BSA was added to the plasma samples on an ACL Pro Elite hemostasis analyzer (Instrumentation Laboratory, USA). Clotting was initiated with 25 mM CaCl₂. The volume ratio of plasma:TF:CaCl₂ was 1:1:1. For data evaluation, clotting time was plotted against the log of the fucoidan concentration, resulting in a sigmoidal curve, which was fitted using SigmaPlot 12
software. Fucoidan concentrations which inhibit 50% of TFPI activity (EC$_{50}$) were derived from the curves.

**Activated partial thromboplastin time (aPTT) assay**

For aPTT assays, 50 µL of thawed normal human plasma pool (George King Biomedical, USA) was mixed with 5 µL of fucoidan sample (0-5 µM final plasma concentration). Fucoids were diluted in imidazole buffer (3.4 g/L imidazole; 5.85 g/L NaCl, pH 7.4) containing 1% albumin (Baxter Innovations GmbH, Austria). After adding 50 µL aPTT reagent (STA APTT, Diagnostica Stago, France), the samples were incubated for 4 min at 37 °C. Clotting was initiated by 50 µL of 25 mM CaCl$_2$ solution (Baxter Innovations GmbH, Austria) and recorded for up to 5 min. All sample processing steps and clotting time measurements were performed with an ACL Pro Elite (Instrumentation Laboratory, USA) instrument. Samples were run in duplicate. The aPTT was then plotted against the fucoidan concentration, and the concentration with a 50% increase in clotting time over baseline was recorded.

**Surface plasmon resonance (SPR) interaction studies**

The interaction of selected fucoids with human full-length TFPI (aa 1 - 276) and a truncated TFPI$_{1-160}$ protein (Baxter Innovations GmbH, Austria) was assessed in SPR experiments (Biacore T200, GE Healthcare, USA). TFPI proteins were covalently coupled to a CM5 chip (GE Healthcare, USA) using conventional amine coupling chemistry resulting in immobilization of 1000 RU response units (RU) for fI-TFPI and 600 RU for TFPI$_{1-160}$. For kinetic analysis, the surfaces were equilibrated with HBS-N buffer (0.01 M HEPES, pH 7.4; 0.15 M NaCl, GE Healthcare, USA) with 0.1% Tween-80 at a flow rate of 30 µL/min. After 60 s, fucoidan samples (0-1.5 µM) dissolved in equilibration buffer were injected for 360 sec followed by a dissociation time of 600 sec. The chip was regenerated by injecting 22.5 µL of 2.5 M NaCl followed by 15 µL of 10 mM NaOH, 1 M NaCl. The flow rate was 30 µL/min. Each sensorogram was referenced against buffer and the blank cell. Binding was evaluated by Biacore T200 Evaluation Software.

**Results**

**Fraction C6 structure elucidation.**

A “C” and an “H” on the labels used in this study denoted the atom, a superscript A or B the residue and a subscript number the position in the saccharide. Fraction C6 was assigned in both COSY and HSQC spectra (Figures 1 A and B). Three-dimensional HSQC-TOCSY (not
shown) was also used to clarify peak assignments. This fraction was composed of at least two kinds of fucose residues, A and B. The chemical shifts, which were calibrated by the internal standard TMSP, are listed in Supplemental Table 2. The chemical shifts of $^A\text{H}_4$ and $^A\text{C}_4$ are 4.60 and 83.9 ppm, respectively. These relatively high chemical shifts indicate O-sulfation at the hydroxyl on $^A\text{C}_4$. The chemical shifts of other positions are relatively low, indicating no substitutions. Thus, A residues are terminal 4-O-sulfated fucose residues. Both $^B\text{H}_4$ and $^B\text{C}_4$ also have large chemical shifts similar to those of the 4-position in the A residue, which indicates sulfation at the 4-position of the B residue. The chemical shift of $^B\text{C}_3$ has a relatively high value (78.8 ppm), unlike that of the corresponding $^B\text{H}_3$ (4.03 ppm), indicating that the 3-position is the linking position. Therefore, B residues are 1-3 linked 4-O-sulfated fucose residues. The structures are shown in the Supplemental Scheme. The cross peaks of residue A in the HSQC spectrum is relatively intense, indicating an abundance of A residues in this fraction. Given that A is a terminal residue, fraction C6 contains a highly branched polysaccharide with a backbone of B residues connected to branches composed of A residues. The ratio (B:A) is $\sim1.4$ indicating that one in every 1.4 B residues has a branch with an A residue. There are multiple possible branch locations; their corresponding NMR signals were highly dispersed and weak. Therefore, the specific sites to which the branches are attached along the backbone could not be assigned. We denoted branch point residues as C. The ratio of residues A:B:C should theoretically be 1:1.4:1.

In the HMBC spectrum (Supplemental Figure 3), no unambiguous correlation was observed between A and B residues across the oxygen on the glycosidic bands. The proton and carbon chemical shifts of position 1 in these two residues are close. The cross peak labeled as $^B\text{C}_3$ - $^A\text{H}_1$ in Figure 2 could be the correlation between $^B\text{C}_3$ and $^B\text{H}_1$ or between $^B\text{C}_3$ and $^A\text{H}_1$, both pairs of which are separated by 3 bonds. However, no further cross peaks, such as that due to $^B\text{C}_3$ - $^A\text{H}_2$ (separated by four bonds), were observed to confirm the relationship of A and B residues. Despite the lack of observed inter-residue correlations, the branching pattern could still be described in that most of the A and B residues are not directly linked to each other, and most of the B residues are not part of the branched chain. Thus, the major structure of this fraction is identified as a 4-O-sulfated, 1-3 linked and highly branched polyfucan, in which most branches are 4-O-sulfated fucose at 2 and/or 4 positions of branch point residues (Supplemental Scheme).
Suppl. Table 1: DS, Mw, and monosaccharide composition of *F.v.* fucoidan and its charge-separated fractions.

<table>
<thead>
<tr>
<th>Fractions</th>
<th>DS (kD)</th>
<th>Mw (kD)</th>
<th>Fucose</th>
<th>Galactose</th>
<th>Mannose</th>
<th>Xylose</th>
<th>Gluturonic Acid</th>
<th>Glucuronic Acid</th>
<th>Mannuronic Acid</th>
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<tr>
<td>C1</td>
<td>0.29</td>
<td>283</td>
<td>25.9</td>
<td>1.8</td>
<td>22.4</td>
<td>26.3</td>
<td>0.6</td>
<td>12.3</td>
<td>10.8</td>
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<td>C2</td>
<td>0.49</td>
<td>121</td>
<td>36.0</td>
<td>16.7</td>
<td>16.2</td>
<td>15.0</td>
<td>0.4</td>
<td>8.6</td>
<td>7.2</td>
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<tr>
<td>C3</td>
<td>0.72</td>
<td>136</td>
<td>85.4</td>
<td>10.3</td>
<td>0.2</td>
<td>3.1</td>
<td>0.1</td>
<td>0.6</td>
<td>0.4</td>
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<td>C4</td>
<td>0.71</td>
<td>161</td>
<td>86.1</td>
<td>10.0</td>
<td>0.0</td>
<td>3.5</td>
<td>0.0</td>
<td>0.2</td>
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<tr>
<td>C5</td>
<td>0.81</td>
<td>151</td>
<td>95.6</td>
<td>4.1</td>
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<td>0.0</td>
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<td>0.1</td>
<td>0.1</td>
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<tr>
<td>C6</td>
<td>0.76</td>
<td>117</td>
<td>97.2</td>
<td>2.7</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.1</td>
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<tr>
<td>Original fucoidan</td>
<td>0.63</td>
<td>124</td>
<td>82.1</td>
<td>6.4</td>
<td>0.3</td>
<td>8.0</td>
<td>0.2</td>
<td>1.1</td>
<td>1.9</td>
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Suppl. Table 2. Chemical shifts of residues in the major structures of fraction C6.

<table>
<thead>
<tr>
<th>Residues</th>
<th>Position 1</th>
<th>Position 2</th>
<th>Position 3</th>
<th>Position 4</th>
<th>Position 5</th>
<th>Position 6</th>
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<td></td>
<td>Proton chemical shifts (ppm)</td>
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<tr>
<td>A</td>
<td>5.11</td>
<td>3.75</td>
<td>4.00</td>
<td>4.60</td>
<td>4.49</td>
<td>1.24</td>
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<tr>
<td>B</td>
<td>5.10</td>
<td>3.83</td>
<td>4.03</td>
<td>4.76</td>
<td>4.49</td>
<td>1.27</td>
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<td></td>
<td>Carbon chemical shifts (ppm)</td>
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<tr>
<td>A</td>
<td>100.4</td>
<td>71.6</td>
<td>71.7</td>
<td>83.9</td>
<td>69.5</td>
<td>18.6</td>
</tr>
<tr>
<td>B</td>
<td>100.9</td>
<td>70.1</td>
<td>78.8</td>
<td>82.5</td>
<td>69.5</td>
<td>18.6</td>
</tr>
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</table>

Suppl. Figure 1: Representative DEAE chromatogram of charge-separated fucoidan.

The odd-numbered tubes were quantified for carbohydrate content by phenol-sulfuric acid assay.

Fraction * was contaminated with glycerol. Therefore, results were not reported.
Supplemental Scheme

Residues A and B

![Chemical structures of residues A and B](image)

Major structures of Fraction C6

![Chemical structures of major structures of Fraction C6](image)

Where \( R_1 = \text{SO}_3\text{Na (8%, mol%)} \) or \( \text{H (92%, mol%)}; \) \( n = 276; m \approx 191 \). These numbers were calculated using the sulfur content and molecular weight of this fraction.
Suppl. Figure 2: $^1$H-$^1$H COSY and $^1$H-$^{13}$C HSQC spectra of fraction C6.

Suppl. Figure 3: HMBC spectrum of fraction C6.
Activation of the contact pathway by fucoidans. Selected fucoidans were tested in the CAT assay in normal human plasma (reference line) with CTI (filled circles) or without CTI (open circles) for their ability to initiate contact activation. A high DS and very high Mw of fucoidan seem to favor this undesired reaction.
Suppl. Figure 5: Overlaid SEC-MALLS chromatograms of size-separated fucoidan.

Brown, black, blue, green, red, and pink curves correspond to DP 840, 590, 200, 70, 50, and 40, respectively.

Suppl. Figure 6: Overlaid $^1$H and $^{13}$C NMR spectra of size-separated fractions.
References


