

Antithrombotic and Antiplatelet Activities of New Isohopane Triterpene from the Roots of *Rubia akane*

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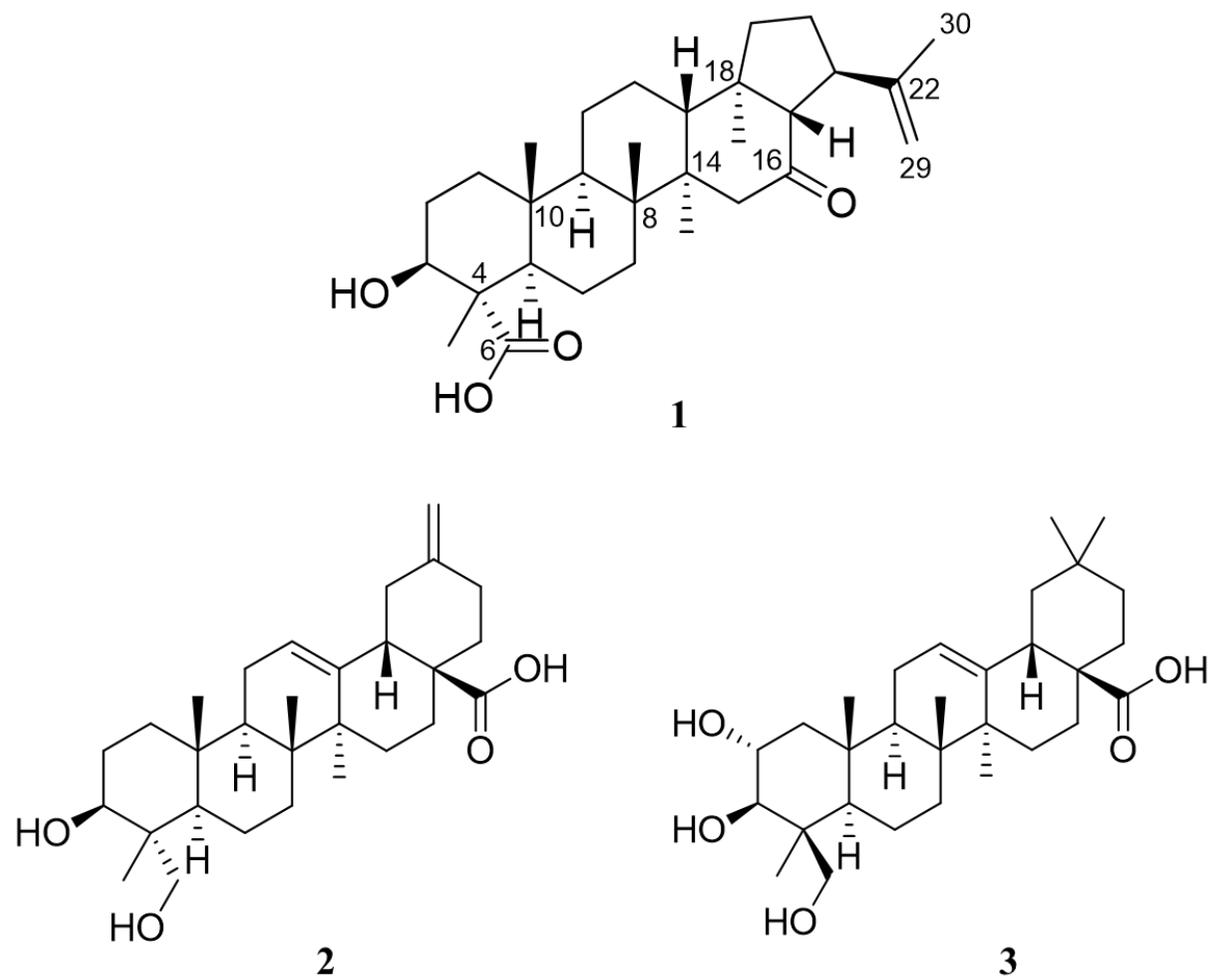


Figure S1. Structures of compounds 1-3

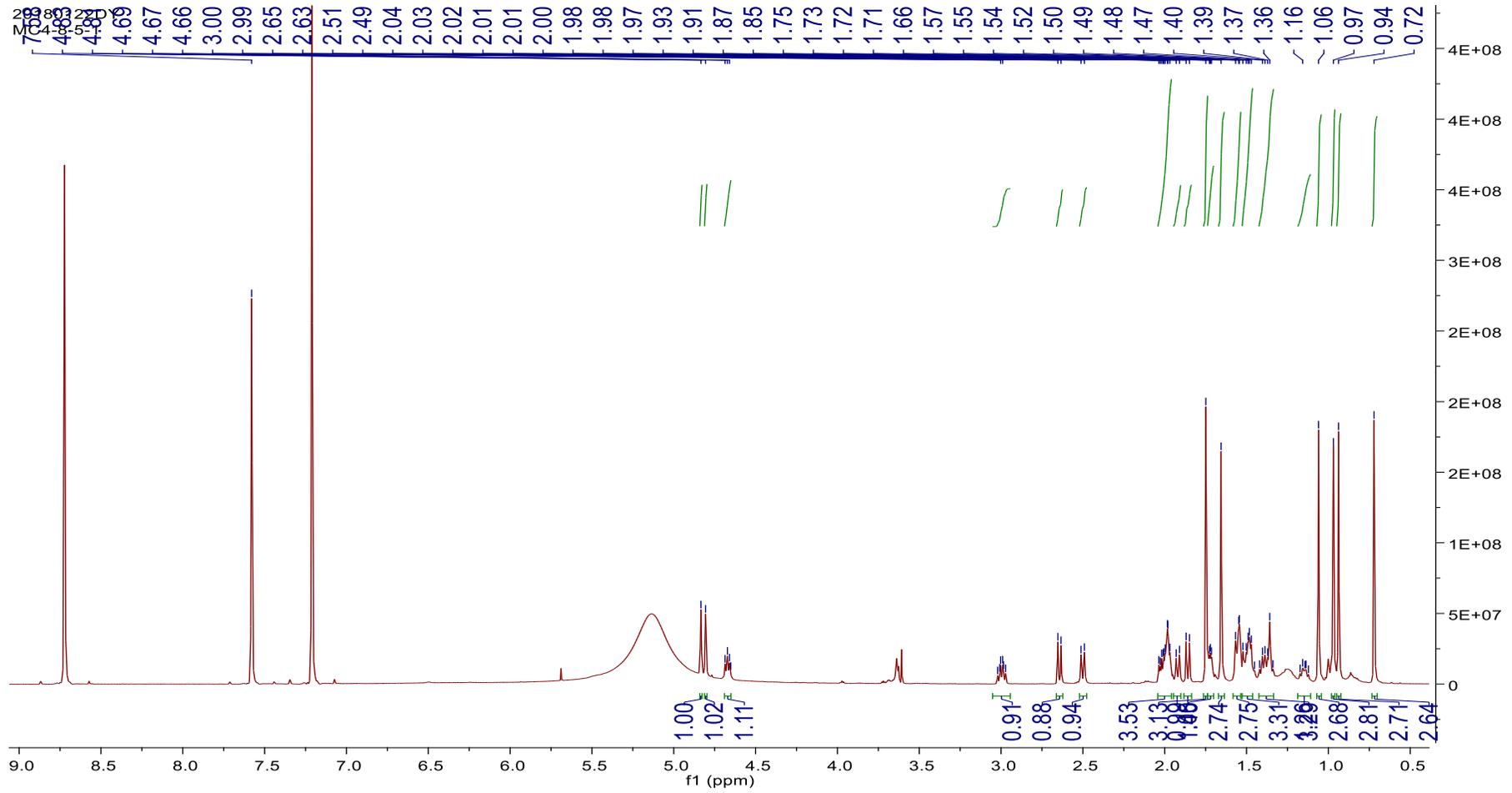


Figure S2. ¹H NMR spectrum of **1** (pyridine-*d*₅, 600 MHz)

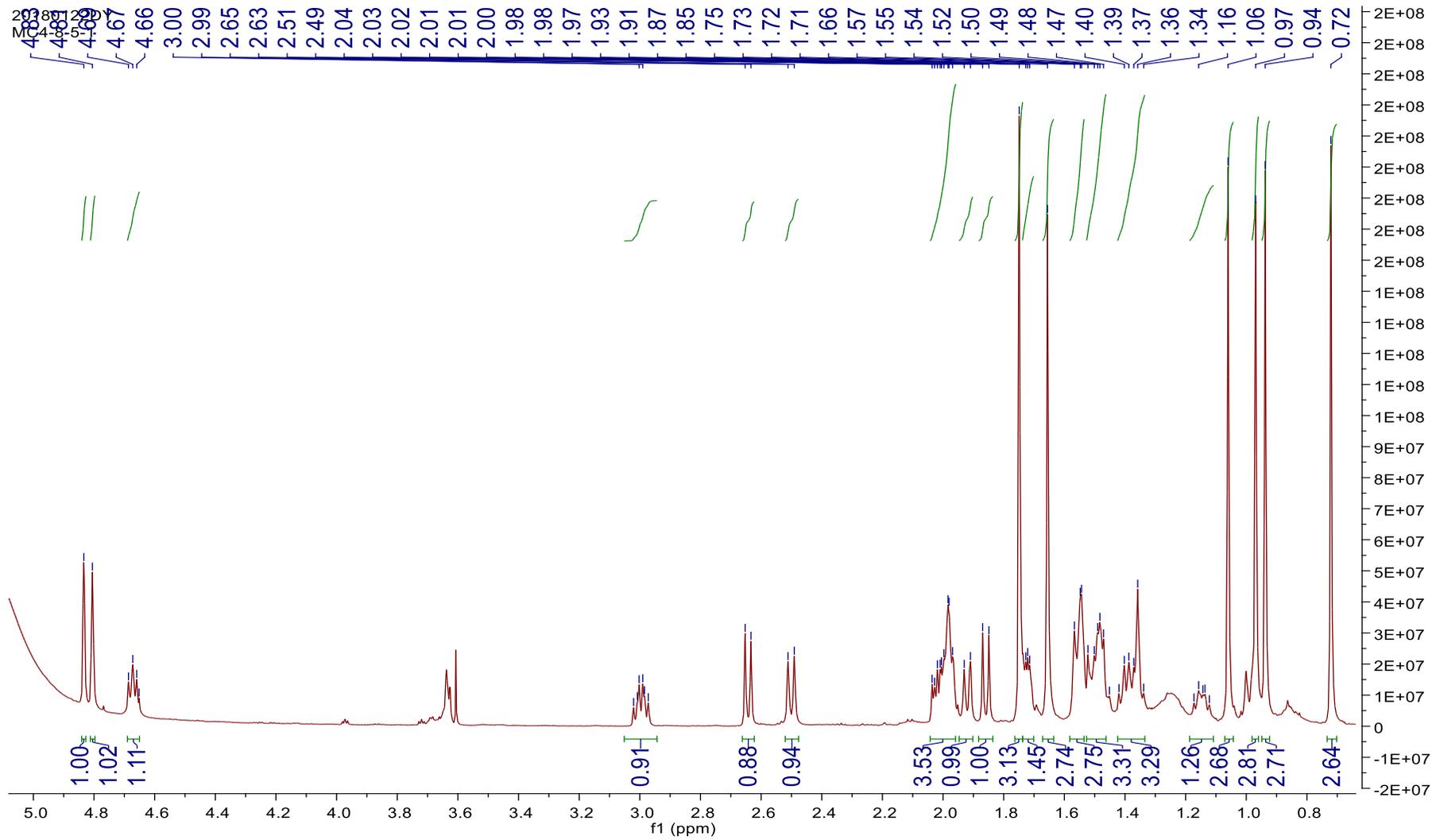


Figure S3. ^1H NMR spectrum of **1** ($\text{pyridine-}d_5$, 600 MHz), expanded

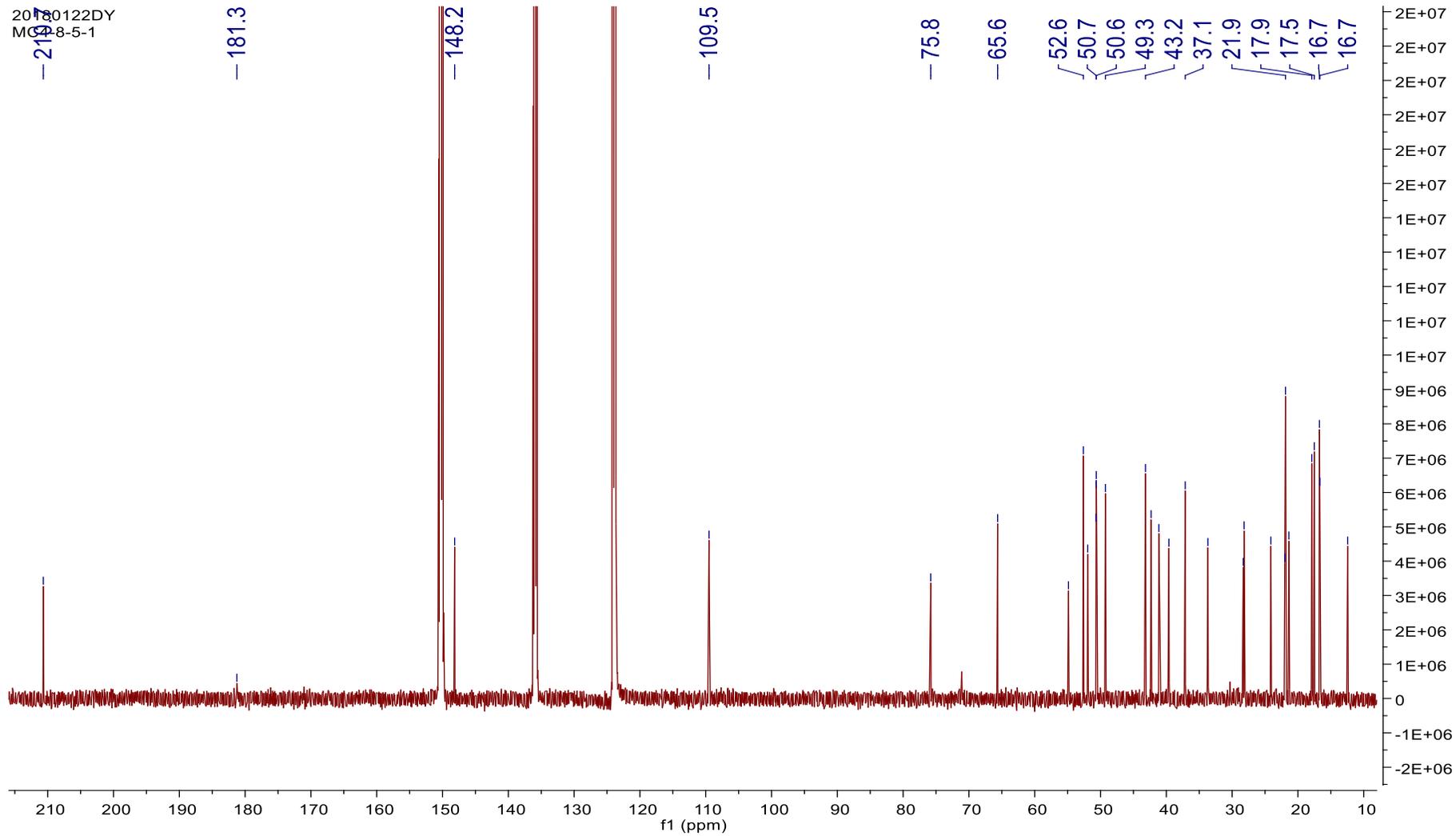


Figure S4. ^{13}C NMR spectrum of **1** (pyridine- d_5 , 600 MHz)

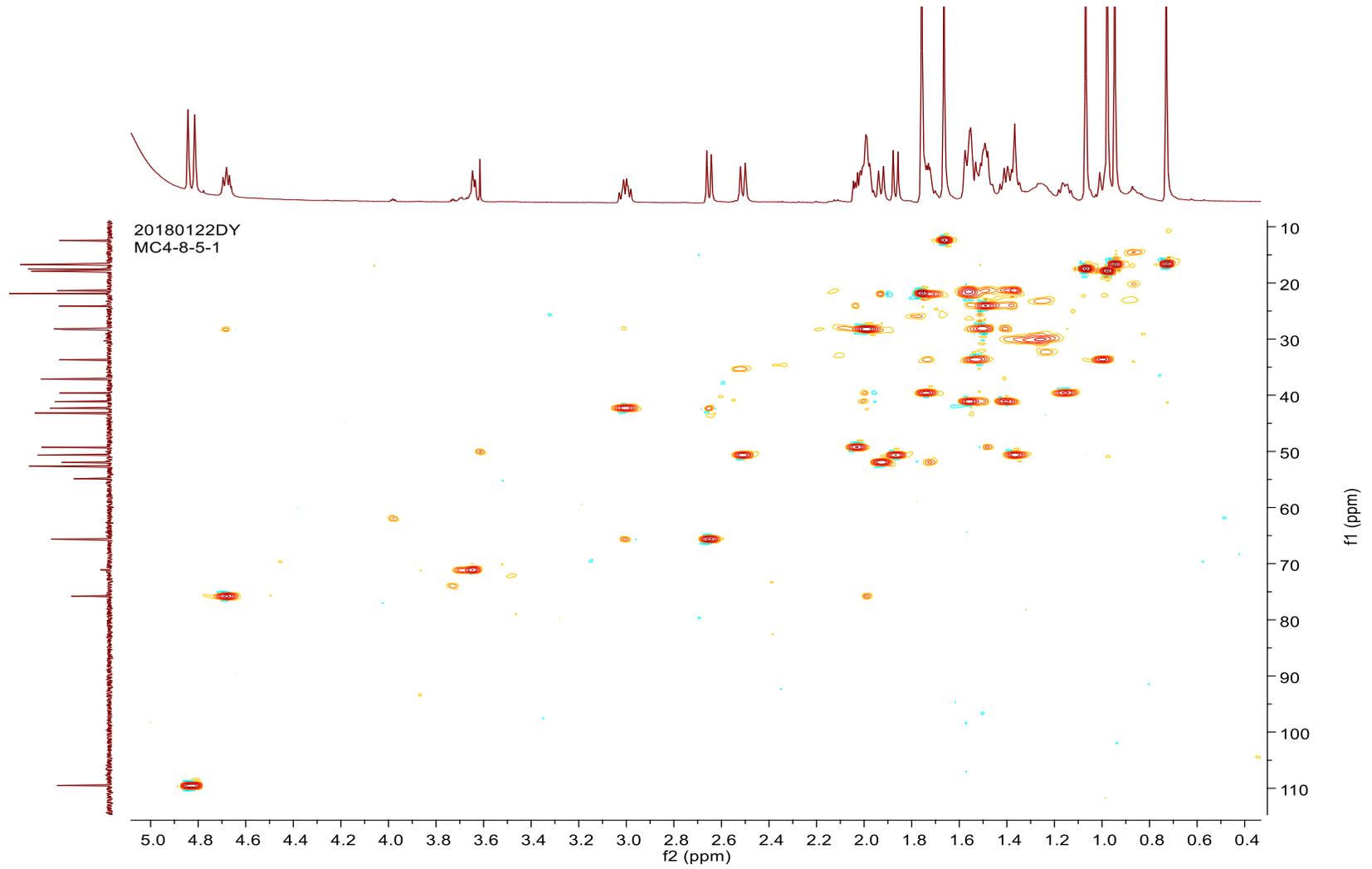


Figure S5. HSQC spectrum of compound **1** in pyridine-*d*₅

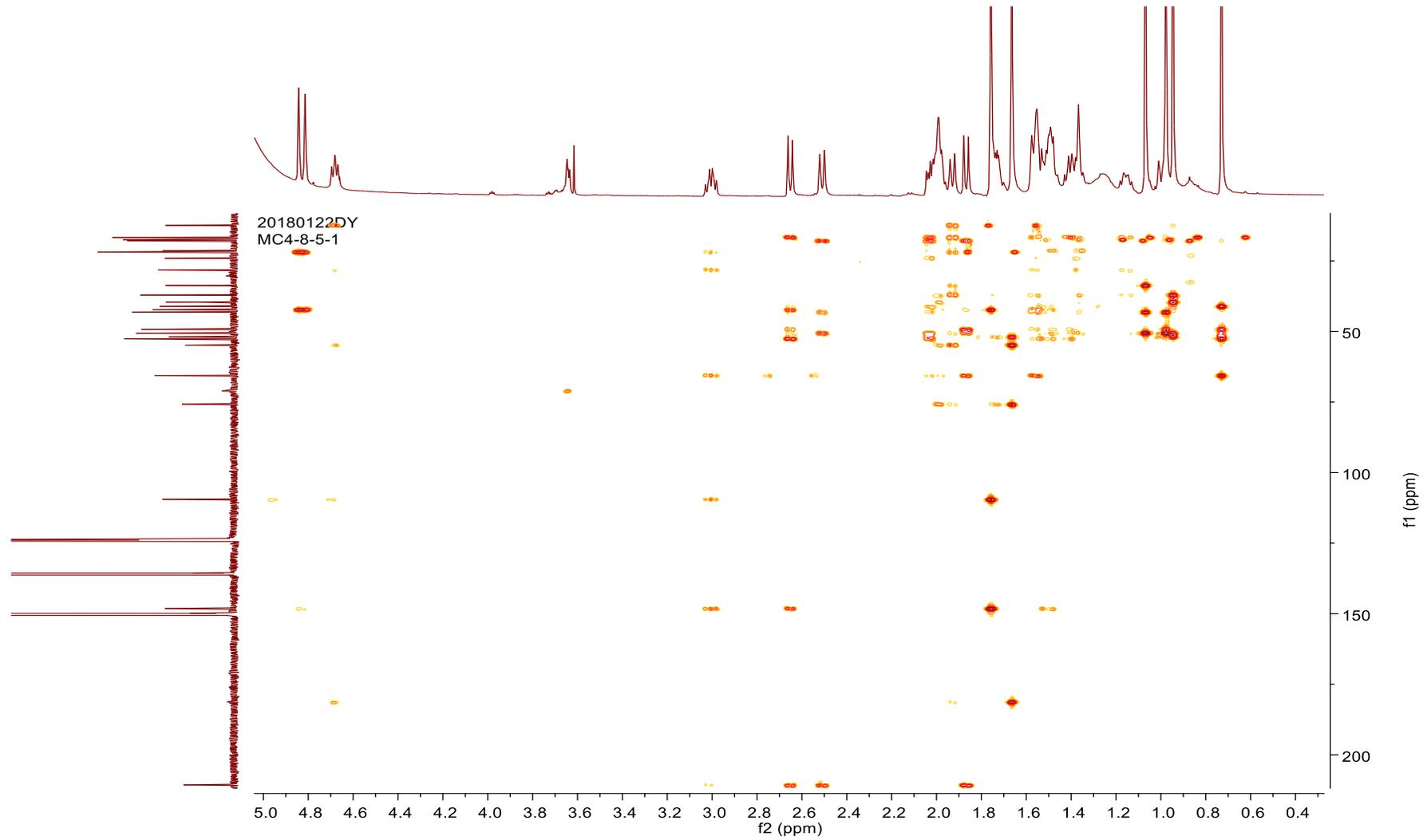


Figure S6. HMBC spectrum of compound **1** in pyridine- d_5

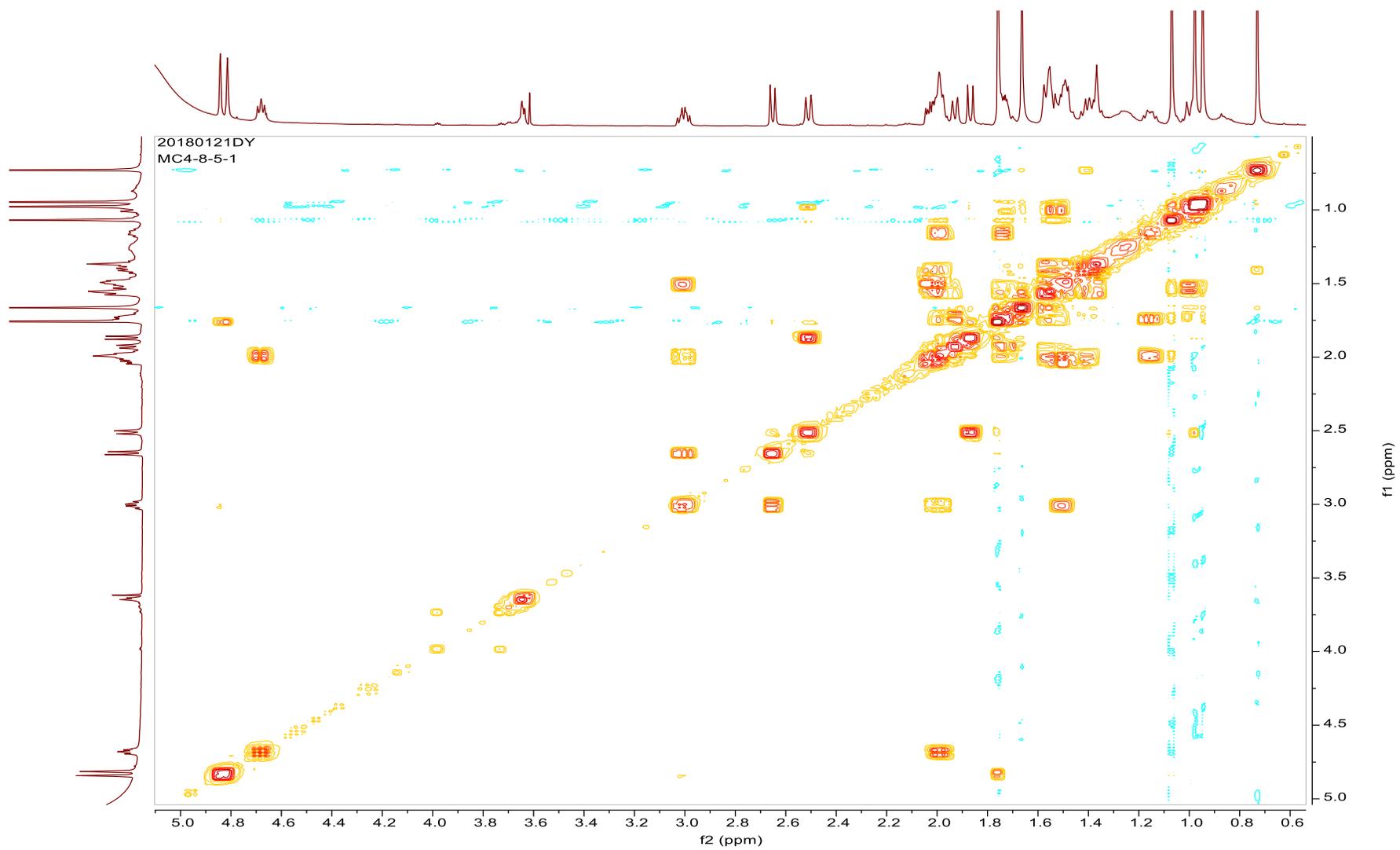


Figure S7. COSY spectrum of compound 1 in pyridine- d_5

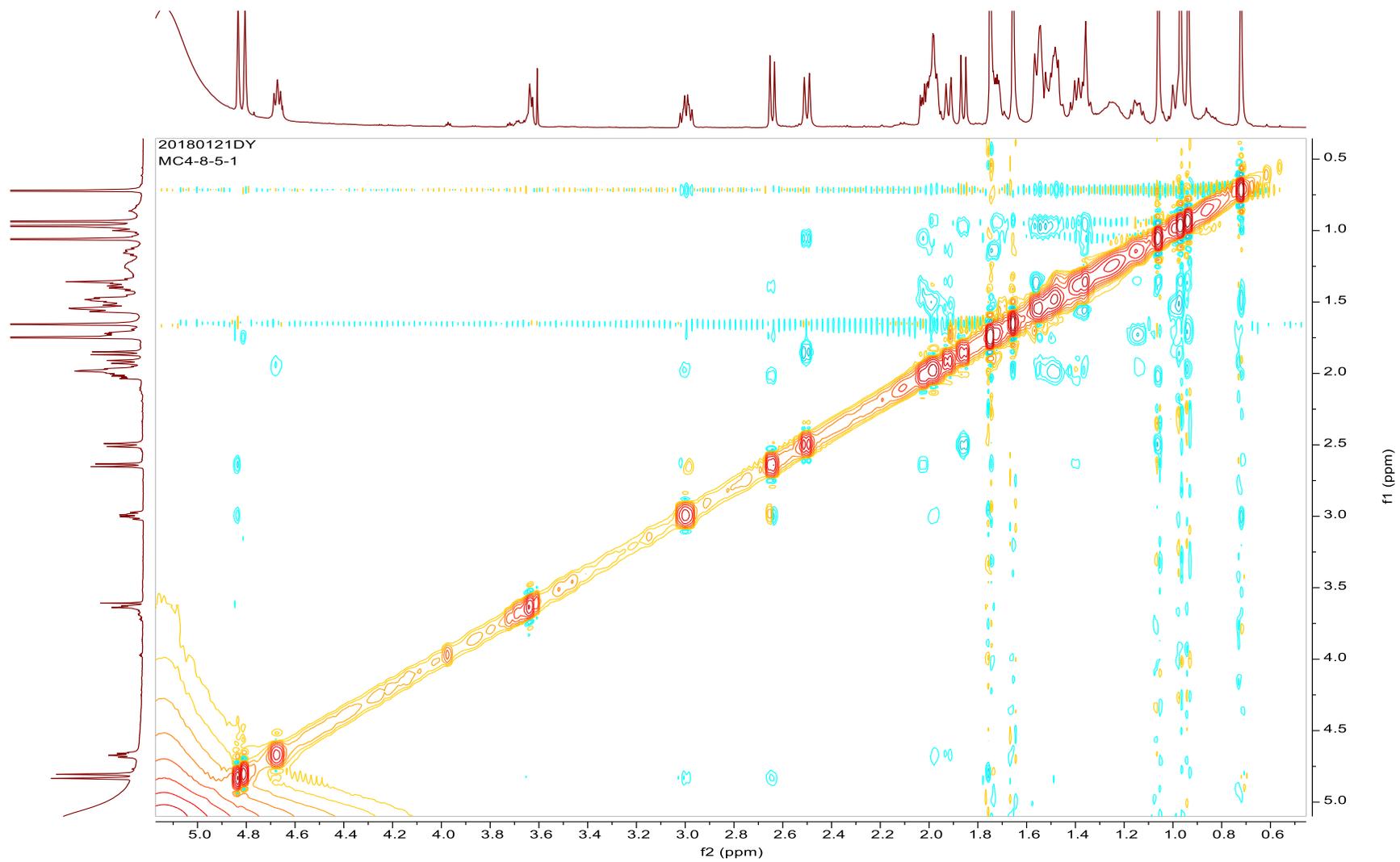


Figure S8. NOESY spectrum of compound **1** in pyridine-*d*₅

Materials and Methods

Reagents

TNF- α was purchased from Abnova (Taiwan). Anti-tissue factor antibody was purchased from Santa Cruz Biologics (Santa Cruz, CA). Factor V, VII, VIIa, FX, FXa, antithrombin III (AT III), prothrombin, and thrombin were obtained from Haematologic Technologies (Essex Junction, VT, USA). aPTT assay reagent and PT reagents were purchased from Fisher Diagnostics (Middletown, Virginia, USA), and the chromogenic substrates, S-2222 and S-2238, were purchased from Chromogenix AB (Sweden). Rivaroxaban (direct FXa inhibitor) and argatroban (direct FIIa inhibitor) were purchased from Santa Cruz Inc. (Dallas, Texas, USA). PAI-1 and t-PA ELISA kits were purchased from American Diagnostica Inc. (Stamford, CT, USA). Other reagents were of the highest commercially available grade.

Isolation of human plasma

Human blood samples were taken in the morning from 10 healthy volunteers in fasting status (aged between 24 and 28 years, four males and six females) without cardiovascular disorders, allergy and lipid or carbohydrate metabolism disorders, and untreated with drugs. All subjects gave written informed consent before participation. Healthy subjects did not use addictive substances or antioxidant supplementation, and their diet was balanced (meat and vegetables). Human blood was collected into sodium citrate (0.32% final concentration) and immediately centrifuged (2000 rpm \times 15 min) in order to obtain plasma.

Anticoagulation assay

aPTT and PT were determined using a Thrombotimer (Behnk Elektronik, Germany), according to the manufacturer's instructions, as described previously [1]. In brief, citrated normal human plasma (90 μ l) was mixed with 10 μ l of **1** and incubated for 1 min at 37°C. aPTT assay reagent (100 μ l) was added and incubated for 1 min at 37°C, followed by addition of 20 mM CaCl₂ (100 μ l). Clotting times were recorded. For PT assays, citrated normal human plasma (90 μ l) was mixed with 10 μ l of **1** stock and

incubated for 1 min at 37°C. PT assay reagent (200 µl), which had been pre-incubated for 10 min at 37°C, was then added and clotting time was recorded. PT results are expressed in seconds and as International Normalized Ratios (INR), and aPTT results are expressed in seconds. $INR = (PT \text{ sample} / PT \text{ control})^{ISI}$. ISI = international sensitivity index.

Platelet aggregation assay

Platelet-rich plasma (PRP) was obtained from syngeneic donor mice by double centrifugation (200 g to create platelet/plasma phase, 500 g to pellet platelets). The platelet-rich plasma was adjusted to a concentration of 1×10^9 platelets/mL with use of a hemocytometer for cell counts. Mouse platelets from platelet-rich plasma (PRP) were washed once with HEPES buffer (5 mM HEPES, 136 mM NaCl, 2.7 mM KCl, 0.42 mM NaH_2PO_4 , 2 mM MgCl_2 , 5.6 mM glucose, 0.1% BSA (w/v), pH to 7.45) in the presence of 1mM CaCl_2 . The platelet aggregation study was carried out according to a method previously reported [2]. Washed platelets were incubated with indicated **1** in TBS for 3min, and then stimulated by thrombin (3U/mL, Sigma) in 0.9% saline solution at 37°C for 5min or collagen (1 µg/mL) at 37°C for 15min. Platelet aggregation was recorded using an aggregometer (Chronolog, Havertown, PA, USA).

Thrombin-catalyzed fibrin polymerization

Thrombin-catalyzed polymerization was determined every 6 s for 20 min by monitoring turbidity at 360 nm using a spectrophotometer (TECAN, Switzerland) at ambient temperature. Control plasma and plasma incubated with **1** were diluted three times in TBS (50 mM Tris-buffered physiological saline solution pH 7.4) and clotted with thrombin (final concentration - 0.5 U/mL). The maximum polymerization rate (V_{max} , $\Delta\text{mOD}/\text{min}$) of each absorbance curve was recorded [3]. All experiments were performed three times.

Cell culture

Primary HUVECs were obtained from Cambrex Bio Science (Charles City, IA) and were maintained using a previously described method [4, 5]. Briefly, cells were cultured until confluent at 37°C at 5% CO₂ in EBM-2 basal media supplemented with growth supplements (Cambrex Bio Science). All experiments were carried out with HUVEC at passage 3–5.

Animals and husbandry

Male C57BL/6 mice (6–7 weeks old, weighing 18–20 g), purchased from Orient Bio Co. (Sungnam, Republic of Korea), were used after a 12-day acclimatization period. Animals were housed five per polycarbonate cage under controlled temperature (20–25°C) and humidity (40–45%) and a 12:12 h light: dark cycle. Animals received a normal rodent pellet diet and water ad libitum during acclimatization. All animals were treated in accordance with the ‘Guidelines for the Care and Use of Laboratory Animals’ issued by Kyungpook National University (IRB No. KNU2017-88).

Cell viability assay

MTT was used as an indicator of cell viability. Cells were grown in 96-well plates at a density of 5×10^3 /well. After 24 h, cells were washed with fresh medium, followed by treatment with 1. After a 48-h incubation period, cells were washed, and 100 μ l of 1 mg/mL MTT was added, followed by incubation for 4 h. Finally, 150- μ l DMSO was added in order to solubilize the formazan salt formed, the amount of which was determined by measuring the absorbance at 540 nm using a microplate reader (Tecan Austria GmbH, Austria). Data were expressed as mean \pm SD of at least three independent experiments.

Factor Xa production on the surfaces of HUVECs

TNF- α (10 ng/mL for 6 h in serum-free medium) stimulated confluent monolayers of HUVECs (preincubated with the indicated concentrations of 1 for 10 min) in a 96-well culture plate were incubated with FVIIa (10 nM) in buffer B (buffer A supplemented with 5 mg/mL bovine serum albumin [BSA] and 5 mM CaCl₂) for 5 min at

37°C in the presence or absence of anti-TF IgG (25 µg/mL). FX (175 nM) was then added to the cells (final reaction mixture volume, 100 µl) and incubated for 15 min. The reaction was stopped by addition of buffer A (10 mM HEPES, pH 7.45, 150 mM NaCl, 4 mM KCl, and 11 mM glucose) containing 10 mM EDTA and the amounts of FXa generated were measured using a chromogenic substrate. Changes in absorbance at 405 nm over 2 min were monitored using a microplate reader. Initial rates of color development were converted into FXa concentrations using a standard curve prepared with known dilutions of purified human FXa.

Thrombin production on the surfaces of HUVECs

Measurement of thrombin production by HUVECs was quantitated as previously described [1, 5]. Briefly, HUVECs were pre-incubated in 300 µl containing **1** in 50 mM Tris-HCl buffer, 100 pM FVa, and 1 nM FXa for 10 min, followed by addition of prothrombin to a final concentration of 1 µM. After 10 min, duplicate samples (10 µl each) were transferred to a 96-well plate containing 40 µl of 0.5 M EDTA in Tris-buffered saline per well in order to terminate prothrombin activation. Activated prothrombin was determined by measuring the rate of hydrolysis of S2238 at 405 nm. Standard curves were prepared using amounts of purified thrombin.

Thrombin activity assay

1 in 50 mM Tris-HCl buffer (pH 7.4) containing 7.5 mM EDTA and 150 mM NaCl was mixed in the absence or presence of 150 µl of AT III (200 nM). Heparins with AT III (200 nM) were dissolved in physiological saline and placed in the sample wells. Following incubation at 37°C for 2 min, thrombin solution (150 µl; 10 U/mL) was added, followed by incubation at 37°C for 1 min. S-2238 (a thrombin substrate; 150 µl; 1.5 mM) solution was then added and absorbance at 405 nm was monitored for 120 s using a spectrophotometer (TECAN, Switzerland).

Factor Xa (FXa) activity assay

These assays were performed in the same manner as the thrombin activity assay, but using factor Xa (1 U/ mL) and S-2222 as substrates.

In vivo bleeding time

Tail bleeding times were measured using the method described by Dejana et al. [1, 6]. Briefly, C57BL/6 mice were fasted overnight before experiments. One h after intravenous administration of Asp or Not, tails of mice were transected at 2 mm from their tips. Bleeding time was defined as the time elapsed until bleeding stopped. When the bleeding time exceeded 15 min, bleeding time was recorded as 15 min for the analysis. All animals were treated in accordance with the Guidelines for the Care and Use of Laboratory Animals issued by Kyungpook National University.

Ex vivo clotting time

Male C57BL/6 mice were fasted overnight and **1** in 0.5% DMSO was administered by intravenous injection. One hour after administration, arterial blood samples (0.1 mL) were withdrawn into 3.8% Na-citrate (1/10; v/v) for ex vivo aPTT and PT determination. Clotting times were performed as described in section 2.2. All animals were treated in accordance with the Guidelines for the Care and Use of Laboratory Animals issued by the Kyungpook National University.

ELISA for PAI-1 and t-PA

The concentrations of PAI-1 and t-PA in HUVEC cultured supernatants were determined using ELISA kits (American Diagnostica Inc. CT, USA).

Statistical Analysis

Results are expressed as mean \pm standard error of the mean (SEM) of at least three independent experiments with duplicate determination. Statistical significance was defined to be based on a p value smaller than 0.05 (SPSS, version 14.0, SPSS Science, Chicago, IL, USA).

Table S1: Anticoagulant activity of compound **1**

| <i>In vitro</i> coagulant assay | | | | |
|---------------------------------|---------------|------------------------|------------|----------|
| Sample | Dose | aPTT (s) | PT (s) | PT (INR) |
| Control | saline | 26.4 ± 0.4 | 12.0 ± 0.6 | 1.00 |
| Comp. 1 | 10 µM | 26.0 ± 0.3 | 12.4 ± 0.2 | 1.07 |
| | 20 µM | 26.3 ± 0.1 | 13.8 ± 0.4 | 1.26 |
| | 50 µM | 26.6 ± 0.3 | 14.7 ± 0.2 | 1.41 |
| | 100 µM | 29.6 ± 0.4* | 20.8 ± 0.6 | 1.94* |
| | 200 µM | 30.6 ± 0.3* | 19.8 ± 0.2 | 1.87* |
| Heparin | 50 µM | 73.1 ± 1.9* | 31.6 ± 0.2 | 8.31* |
| Warfarin | 50 µM | 69.2 ± 0.7* | 29.8 ± 1.0 | 7.18* |
| <i>In vivo</i> bleeding time | | | | |
| Sample | Dose | Tail Bleeding time (s) | n | |
| Control | Saline | 34.1 ± 1.2 | 10 | |
| Comp. 1 | 18.8 µg/mouse | 33.6 ± 1.7* | 10 | |
| | 47.0 µg/mouse | 35.4 ± 2.1* | 10 | |
| | 94.0 µg/mouse | 47.3 ± 2.2* | 10 | |

| Heparin | 360.0 µg/mouse | 88.3 ± 2.8* | 10 | |
|---------------------------------|----------------|-------------|------------|----------|
| Warfarin | 30.8 µg/mouse | 82.6 ± 2.9* | 10 | |
| <i>Ex vivo</i> coagulation time | | | | |
| Sample | Dose | aPTT (s) | PT (s) | PT (INR) |
| Control | saline | 30.6 ± 1.0 | 12.7 ± 1.1 | 1.00 |
| Comp. 1 | 18.8 µg/mouse | 31.0 ± 0.8 | 11.8 ± 1.7 | 1.02 |
| | 47.0 µg/mouse | 35.4 ± 1.6* | 14.4 ± 1.8 | 1.22 |
| | 94.0 µg/mouse | 47.4 ± 1.5* | 21.4 ± 1.6 | 2.44* |

^aEach value represents the means ± SEM (n=5).

* p < 0.05 as compared to control.

Effects of compound 1 on production of thrombin and FXa: In the current study, pre-incubation of HUVECs with FVa and FXa in the presence of CaCl₂ prior to addition of prothrombin resulted in production of thrombin (Figure 3C). In addition, treatment with **1** or Not resulted in dose-dependent inhibition of production of thrombin from prothrombin (Figure 3C). According to findings reported by Rao et al., the endothelium provides the functional equivalent of pro-coagulant phospholipids and supports activation of FX [7], and, in TNF- α stimulated HUVECs, activation of FX by FVIIa occurred in a TF expression-dependent manner [8]. Thus, we investigated the effects of **1** on activation of FX by FVIIa. HUVECs were stimulated with TNF- α for induction of TF expression, and, as shown in Figure 3D, the rate of FX activation by FVIIa was 15-fold higher in stimulated HUVECs (92.2 ± 4.5 nM) than in non-stimulated HUVECs (5.8 ± 2.2 nM), and this increase in activation was abrogated by anti-TF IgG (18.1 ± 2.9 nM). In addition, pre-incubation with **1** resulted in dose-dependent inhibition of FX activation by FVIIa (Figure 3D). Therefore, these results suggest that **1** can inhibit production of thrombin and FXa.

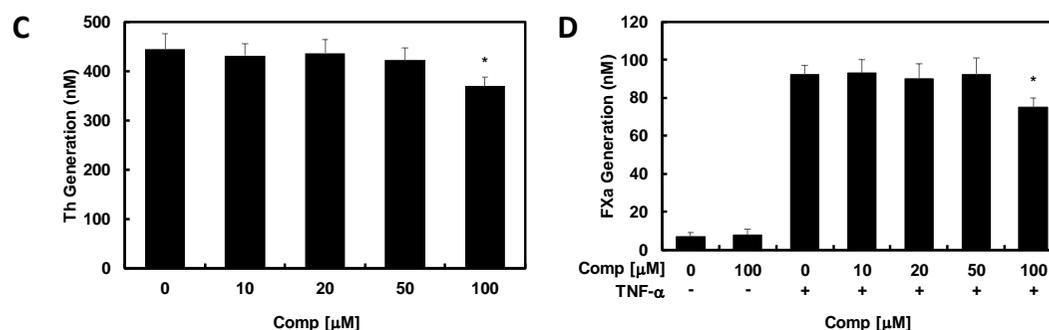


Figure S9 Effects of **1** on inactivation and production of thrombin and factor Xa. (C) HUVEC monolayers were pre-incubated with FVa (100 pM) and FXa (1 nM) for 10 min with the indicated concentrations of **1**. Prothrombin was added to a final concentration of 1 μ M and prothrombin activation was determined 30 min later. (D) HUVECs were pre-incubated with the indicated concentrations of **1** for 10 min. TNF- α (10 ng/mL for 6 h) stimulated HUVECs were incubated with FVIIa (10 nM) and FX (175 nM) in the absence or presence of anti-TF IgG (25 μ g/mL) and FXa production was determined. D=0.2% DMSO is the vehicle control. * $p < 0.05$ vs. 0.0001 D (C) or TNF- α alone (D).

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