The components and activities analysis of noval anticoagulant candidate dHG-5

Huifang Sun[1], Na Gao[2], Lin Ren[1], Shuang Liu[1], Lisha Lin[1], Wenqi Zheng[2], Lutan Zhou[1], Ronghua Yin[1], and Jinhua Zhao[1]

[1]Kunming Institute of Botany Chinese Academy of Sciences
[2]South-Central University for Nationalities

April 28, 2020

Abstract

Background and purpose: intrinsic Xase (iXase) is the final and rate-limiting enzyme complex in the intrinsic coagulation pathway, which may be the target for anticoagulation and anti-thrombosis with lower bleeding tendency. A depolymerized fucosylated glycosaminoglycan (dHG-5) with molecular weight of 5.2 kDa was prepared from sea cucumber Holothuria fuscopunctata, showed promising druggability as an anticoagulant targeting iXase. Like heparin, the drug candidate dHG-5 is composed of a series of oligosaccharides. Therefore, it is necessary to clarify these oligosaccharides' structures and contents in dHG-5, and their contribution to the pharmacological activities of dHG-5. Experimental approach: These oligosaccharides' structures were confirmed by 2D NMR and MS spectra. The coagulation factor inhibition and factor IXa-binding activities were analyzed by chromogenic substrates and BLI, respectively. Anticoagulation was evaluated by clotting time. Antithrombotic activity and bleeding risk were evaluated by deep venous thrombosis model and tail-bleeding model, respectively. Key results: Our data revealed that 1) the nine purified oligosaccharide components were homologous and shared the common formula, which accounted for about 95% of dHG-5, 2) the relationships of anti-iXase, f.IXa-binding, APTT-prolonging and antithrombotic activity potenies (y) and oligosaccharides' molecular weight (x) fitted well with power function ($y = a \times x^b$), 3) the activity potencies of dHG-5 were approximately equivalent to the weighted average sum of those of its oligosaccharides, 4) dHG-5 showed antithrombotic activity with low bleeding tendency and predictable pharmacodynamics characteristic. Conclusion and Implications: dHG-5 has antithrombosis with low bleeding tendency, and clear chemical composition and pharmacological properties, which makes a good preparation for the clinical study.

Key words: oligosaccharide, iXase inhibitor, anticoagulation, antithrombosis, bleeding risk

Word count: 245

Abbreviations

APTT = activated partial thromboplastin time

AT-III = antithrombin III

$AUC_{(0–8)}$ = area under curve within 8 hours

BSA = bovine serum albumin

BLI = bio-layer interferometry

dFG = depolymerized FG

dp = degree of polymerization

DS = dermatan sulfate

$EC_{2.0\times}$ = oligosaccharides concentration required to double clotting time

ED_{50} = 50% effective dose

E_{max} = the maximum effect

ESI-Q-TOF MS = negativeion electrospray ionization quadrupole time-of-flight mass spectrometry

f.VII = factor VII

f.VIII = factor VIII

f.X = factor X

f.XII = factor XII

f.IIa = activated factor II, thrombin

f.VIIa = activated f.VII

f.VIIIa = activated factor VIII

f.IXa = activated factor IXa

f.Xa = activated factor Xa

f.XIa = activated factor XIa

f.XIIa = activated factor XII

FG = fucosylated glycosaminoglycan

FucS = sulfated fucose

Fuc_{3S4S} = 3,4-di-O-sulfated-fucose

Fuc_{2S4S} = 2,4-di-O-sulfated-fucose

Fuc_{4S} = 4-O-sulfated-fucose

GalNAc = N-acetyl galactosamine

GlcA = glucuronic acid

GPC = gel permeation chromatography

HCII = heparin cofactor II

HPGPC = high performance gel permeation chromatography

iXase = intrinsic Xase, i.e., the f.IXa-f.VIIIa complex

IC_{50} = the concentration of compounds inhibiting 50% protease activity

LMWH = low-molecular-weight heparin

Mw = molecular weight

MWCO = molecular weight cutoff

NS = normal saline

OD_{405nm} = optical density at 405 nm

OSCS = oversulfated chondroitin sulfate

2

PEG = polyethylene glycol

PPP = poor platelets plasma

PRP = platelets rich plasma

PT = prothrombin time

SD = standard deviation

$t_{1/2}$ = half life

TF = tissue factor

TT = thrombin time

WP = washed platelets

$\Delta OD_{405nm}/min$ = change rate of absorbance at 405 nm

ΔU = unsaturated uronic acid, 4-deoxy-α-L-threo-hex-4-enopyranosyluronic acid

Bullet point summary

What is already known:

dFG-5 showed potent anti-iXase and antithrombotic activity without effects on f.XII and platelets

What this study adds:

- Oligosaccharides' structures and contents in dHG-5 and contribute to the pharmacological activities of dHG-5
- Pharmacological characteristics of oligosaccharides contained in dHG-5

Clinical significance:

The selective iXase inhibitor dHG-5 is a potential candidate for treating venous thrombosis

Introduction

Cardiovascular disease has been causing high morbidity and mortality in worldwide (Benjamin, Virani *et al.* , 2018; Timmis, Townsend*et al.* , 2017). Among which, venous thromboembolism, such as deep venous thrombosis and pulmonary embolism, is one of the main complications of major surgery and cancer, *et al* , which remains to be a global disease burden (Becattini, Cohen *et al.* , 2016; Carpenter, Richardson *et al.* , 2018; Jiménez, Bikdeli *et al.* , 2019).

According to the classic coagulation cascade model, intrinsic coagulation pathway is closely involved in pathological thrombosis while dispensable in physiological hemostasis (Colman, 2006; Qiufang, Tucker*et al.* , 2010; Wheeler & Gailani, 2016; Woodruff, Xu *et al.* , 2013). It is initiated by polyanions such as polyphosphate and endotoxin produced in infection processes (Campello, Henderson *et al.* , 2018; Long, Kenne *et al.* , 2016). In the cell-based coagulation model, pathological thrombosis might also be initiated by blood-born tissue factor (TF) (e.g., TF derived from monocytes) under pathological conditions such as inflammation (Hoffman, 2003). After the initiation of coagulation by TF, f.VIII (factor VIII) and platelets are activated by trace amount of thrombin produced in the extrinsic coagulation pathway. Then f.VIIIa (activated factor VIII) forms complex with f.IXa (activated factor IX) in the surfaces of activated platelets, which is essential in the amplification and propogation phases of coagulation, and thus thrombosis (Smith, 2010). The f.VIIIa-f.IXa complex is also known as intrinsic Xase (iXase), the last and rate-limiting enzyme in the intrinsic coagulation pathway (Ahmad, Rawala-Sheikh *et al.* , 1992).

Based on the above theries, selectively inhibiting the intrinsic coagulation pathway is recognized to be a promising strategy for safe antithrombotic therapy (Wheeler & Gailani, 2016). In recent years, inhibitors targeting activated factor XII (f.XIIa), XI (f.XIa) or IX (f.IXa) are being developed, and showed promising

preclinical results (Pinto, Orwat *et al.* , 2017; Quan, Pinto *et al.* , 2018; Robert, Bertolla *et al.* , 2008; Wang, Beck *et al.* , 2010). While anti-iXase drug candidate has not been reported, according to both coagulation model, it should be a good target for effective and safe antithrombosis (Lin, Zhao *et al.* , 2020).

Fucosylated glycosaminoglycan (FG) from sea cucumbers, consisting of the chondroitin sulfate-like backbone and the unique branches of sulfated fucoses, is a potent iXase inhibitor considered as a lead compound for novel anticoagulant (V. H. Pomin, 2014). Native FG exhibited potent anticoagulant and antithrombotic activities, but also had the undesired effects of platelets aggregation and factor XII (f.XII) activation (Fonseca, Santos *et al.* , 2009; J. Z. Li, Bao *et al.* , 1985). Depolymerized FG (dFG) with average molecular weight (Mw) less than 12 kDa showed significantly improved selectivity of anti-iXase activity and had no obvious effects on platelets and f.XII (Kitazato, Kitazato *et al.* , 1996; Wu, Wen *et al.* , 2015). Among them, a 5.2 kDa-dFG from*Holothuria fuscopunctata* (dHG-5) was developed in our group as a promising anticoagulant candidate, due to its advantages in mature preparation process, anti-iXase inhibitor selectivity, antithrombotic activity (Zhou, Gao *et al.* , 2020).

Like the heterogenous polysaccharide heparin, dHG-5 is a multi-component compound and composed of a series of oligosaccharides. However, it's unclear that these oligosaccharides' structures and contents contained in dHG-5, and how these oligosaccharides contribute to the pharmacological activities of dHG-5. We now aimed 1) to purify and identify these oligosaccharide components, 2) to characterize the pharmacological activities of these oligosaccharide components, 3) to reveal the contribution of these oligosaccharide components to dHG-5 in pharmacological behaviors. Besides, we have systematically evaluated the pharmacological activities of dHG-5 in target selectivity, anticoagulation, antithrombosis, bleeding risk and pharmacodynamics.

Animals, materials and methods

All experiments were approved by the regional board (Kunming Institute of Botany, Chinese Academy of Sciences) in accordance with the Chinese animal welfare law, as well as in accordance with local regulations and the NRC Guide for the Care and Use of Laboratory Animals and approved by the local Institutional Animal Care and Use Committee (the Animal Ethics Committee of Kunming Institute of Botany, Chinese Academy of Sciences, No. KIB-SMP-019).

Animals

Sprague-Dawley rats (male, 180 ~ 220g, License No. SCXK 2016-0002) and Kunming mice (male, 18 ~ 22 g, License No. SCXK 2016-0002) were all purchased from Hunan SJA Laboratory Animal Co., Ltd (China). Animals were kept under controlled conditions (12 h dark/12 h light cycle, water and food supply ad libitum). For the characterization of thrombosis inhibition of compounds in rats deep venous thrombosis model, either compound or vehicle (normal saline, NS) was administered subcutaneously after randomization (n=6 respectively). For studying bleeding risk of compounds in mice tail-bleeding model, either compound or NS was administered subcutaneously after randomization (n=8 respectively). For characterizing pharmacodynamics of dHG-5 in mice, dHG-5 was administered subcutaneously after randomization (n=6 respectively).

Materials

Dried sea cucumbers *Holothuria fuscopunctata* were purchased from local markets in Zhanjiang, Guangdong Province, China. Amberlite FPA98Cl ion exchange resin was purchased from Rohm and Haas Company (USA). Polyacrylamide gels (Bio-Gel P2, P6 and P10) were from Bio-Rad (Bio-Rad Laboratories). Sephadex G-25 and G-10 were from GE Healthcare. Deuterium oxide (99.99%) was from Sigma Aldrich. LMWH (Enoxaparin, 0.4 mL 4000 AXaIU, Mw ~4.5 kDa) was from Sanofi-Aventis (France). Dermatan sulfate (DS, Mw 41.4 kDa) was from Sigma-Aldrich (USA). Oversulfated chondroitin sulfate (OSCS) was from National Institutes for the Control of Pharmaceutical and Biological Products (China). The activated partial thromboplastin time (APTT), prothrombin time (PT), thrombin time (TT) reagents, and human coagulation control plasma were all purchased from Teco Medical (Germany). Biophen f.VIII: C kit, Biophen antithrombin III (AT-III)-IIa kit, Biophen AT-III-Xa kit, Biophen VII kit, human AT-III, human factor VII (f.VII), human activated factor X (f.Xa), human thrombin (activated factor II, f.IIa), thrombin chromogenic substrate CS-

4

01(38) and kallikrein chromogenic substrate CS-31(02), CS 11(65) were all from Hyphen BioMed (France). Recombinant coagulation f.VIII was from Bayer HealthCare LLC (USA). Human AT-III deficiency plasma was from Sekisui Diagnostics (USA). Human heparin cofactor II (HCII) deficient plasma was from Affinity Biologicals Inc. (USA). Human f.XIIa and activated factor VII (f.VIIa) was from Enzyme Research Laboratories (USA). Human f.XIa was from Assaypro (USA). Human f.IXa and human factor X (f.X) were from Haenatologic Technologies (USA). S-2366 was from Chromogenix (Italy). Pefchrome f.IXa and f.VIIa was from DSM (Netherlands). Hirudin was from Tian Enze Gene Technology (China), Amine-PEG 3 -Biotin was from MedChemExpress LLC (USA). SA biosensor was from Fortébio (USA). All other chemical reagents were of analytical grade and obtained commercially.

Methods

General Chemistry Methods. All reactions were carried out using commercial grade reagents and solvents. NMR chemical shifts (δ) are reported in parts per million (ppm).

The purity of the oligosaccharide components were determined by HPGPC equipped with a TSKgel G2000SW XL column (7.5 mm × 300 mm) from TOSH Bioscience. The elution conditions for this HPGPC analysis have been described previously.(Shang, Gao *et al.* , 2018; Zhao, Wu *et al.* , 2015) The columns were eluted with carefully degassed 0.2 M sodium chloride solution at a flow rate of 0.4 mL/min. The HPLC series were equipped with a refractive index detector (RID) and diode-array detector (DAD), and the DAD was set at 234 nm.

The Mw of dHG-5 and its distribution were determined by HPGPC with the TSK G2000SW$_{XL}$ column. The five homogeneous oligosaccharides (oHG-5, oHG-8, oHG-11, oHG-14 and oHG-17) were used as standard samples, their peak molecular weights were obtained from the MS. Oligosaccharide retention times - peak molecular weights curve was fitted by third-order polynomial using GPC software, and the Mw of dHG-5 was calculated using the same GPC software.

The oligosaccharide structures were characterized by ^{1}H /^{13}C NMR and 2D NMR spectroscopy, which was performed in D$_2$O at 298 K on a Bruker 800 MHz spectrometer with Topspin 3.2 software, as previously described.(Shang, Gao *et al.* , 2018) All spectra were recorded with HOD suppression by presaturation. The purified oligosaccharide samples (15-25 mg) were dissolved in 1.0 mL 99.9% D$_2$O (Sigma) and lyophilized three times to remove the exchangeable protons. Finally, the samples were re-dissolved in 0.5 mL D$_2$O and transferred to NMR microtubes (outside diameter, 5 mm, Norrell).

The 1D (^{1}H /^{13}C) and 2D (^{1}H-^{1}H COSY, TOCSY, NOESY, and ^{1}H-^{13}C HSQC, HMBC) NMR spectra were recorded under conditions described earlier (Cai, Yang *et al.* , 2019) with minor modifications and processed using a trial MestReNova v9.0.1-13254 software.

The MS spectra analysis was conducted on a micrOTOF-QII mass spectrometer (Bruker Daltonic, Germany) equipped with an electrospray ion source (ESI). The fractionated and desalted samples were dissolved in 100% pure water and introduced into an ion source at a flow rate of 5 µL/min. The MS spectrometric conditions were as follows: ESI in negative ionization mode, capillary voltage of 2500 V, nebulizer pressure of 0.8 bar, drying gas flow rate of 5.0 L/min, and drying gas temperature of +180 °C. The mass spectra of the oligosaccharides were acquired in scan mode (m/z scan range 400-3000). Data analysis was performed using Bruker Compass Data-Analysis 4.0 software (Bruker Daltonic, Germany).

Preparation of dHG-5. The native FG (HPLC purity 99.9%; average molecular mass 45 kDa) was isolated and purified from the sea cucumber *Holothuria fuscopunctata* as previously described.(X. M. Li, Luo *et al.* , 2017) Briefly, the 30 kg dried body wall of the sea cucumber was digested by papain (EC 3.4.22.2) and the polysaccharide components were released by 0.25 M sodium hydroxide. The crude polysaccharide was obtained by repeated salting-out with KOAc and precipitation by ethanol, which was further purified by strong anion-exchange chromatography using FPA98 resin. Finally, about 246 g of native FG was obtained with 0.8 % yield and further used for chemical cleavage to prepare its low molecular weight fragments.

The reaction principle was based on a β-eliminative cleavage of FG through its activated benzyl ester deriva-

tive according to previously research.(Na Gao, Lu *et al.* , 2015) Approximately 130 g of sodium FG was dissolved in 1.9 L of distilled water and completed transalification with benzethonium salts. FG benzethonium salts (325 g) were obtained by precipitation and centrifugation and dried under vacumm condition at room temperature. The FG benzethonium salts were dissolved in 1.6 L N, N-dimethyl formamide (DMF) and esterified by 32 mL benzyl chloride (~2 equals of carboxyl groups in native FG) under continually stirring at 35 °C for 24 h. Then the FG in the DMF solution was cooled to 25°C and depolymerized by adding 550 mL freshly prepared 0.08 M EtONa in ethanol. About 2.2 L saturated sodium chloride solution and 24 L of ethanol were added to the reaction solution successively to complete the transalification of benzethonium salt to sodium salts. Particularly, the saponification procedure in alkaline solution was necessary to hydrolyze the residual benzyl esters, and the reducing ends were reduced to its alcoholic hydroxyl by $NaBH_4$. The depolymerized product was isolated using tangential flow filtration on a Pellicon Mini device equipped with a 0.1 m^2 PLCTK membrane with a molecular weight cutoff (MWCO) of 3 kDa (Millipore) or 10 kDa. The fraction with Mw higher than 3 kDa and lower than 10 kDa were collected and freeze-dried, finally ~30 g dHG-5 was obtained.

Purification of oligosaccharides from dHG-5. The oligosaccharides contained in dHG-5 were fractionated by GPC on a column packed with Bio-gel P6 (fine, 2×150 cm, Bio-Rad) or Bio-gel P10 (medium, 2×200 cm, Bio-Rad). dHG-5 (0.8 g) was dissolved in 10 mL of deionized water, subjected to the Bio-Gel P10 column equilibrated well by 0.2 M NaCl and then eluted with the same solution. The flow rate was approximately 12 mL/h and 100 fractions (2 mL/tube) were collected. Absorbance of each fraction were measured at 234 nm. The oligosaccharide fractions were collected and then desalted by a column packed with Sephadex G-10 (1.5×100 cm). The elution volume - absorbance was plotted to show the oligosaccharide distribution in eluted fractions, those containing the uniform oligosaccharide were pooled (as detected by HPGPC) and lyophilized after repeated fractionating. Five sized homogeneous oligosaccharides from dHG-5 were desalted on a Bio-Gel P-2 column (fine, 1.5 ×100 cm, Bio-Rad) and were freeze-dried, the obtained white powders were compound oHG-5 (pentasaccharide), oHG-8 (octasaccharide), oHG-11 (hendecasaccharide), oHG-14 (tetradecasaccharide) and oHG-17 (heptadecasaccharide). Similarly, another four larger components were obtained and identified as oHG-20 (eicosasaccharide), oHG-23 (tricosasaccharide), oHG-26 (hexacosasaccharide) and oHG-29 (nonacosasaccharide).

oHΓ-5, πεντασαςςηαριδε,Λ-Φυς₃Σ₄Σ-α(1,3)-Λ-Δ^{4,5}ΓλςΑ-α(1,3)-Δ-ΓαλΝΑς₄Σ₆Σ-β(1,4)-[Λ-Φυς₃Σ₄Σ-α(1,] 3)-Δ-ΓλςΑ-oλ:^{1}H NMR (800 MHz, D₂O) δ 5.693 (1H, d, H$_{[?]U}$-4, $J_{(3,4)}$ = 2.40 Hz), 5.201 (1H, d, H$_{dF}$-1, $J_{(1,2)}$ = 3.78 Hz), 5.039 (1H, d, H$_{rF}$-1,$J_{(1,2)}$ = 3.96 Hz), 4.918 (1H, m, H$_A$-4, $J_{(3,4)}$ = 2.16 Hz), 4.859 (1H, d, H$_{[?]U}$-1, $J_{(1,2)}$ = 8.64 Hz), 4.831 (1H, m, H$_{rF}$-4, $J_{(3,4)}$ = 2.76 Hz), 4.831 (1H, m, H$_{dF}$-4,$J_{(3,4)}$ = 2.82 Hz), 4.639 (1H, d, H$_A$-1, $J_{(1,2)}$ = 8.52 Hz), 4.546 (1H, dd, H$_{rF}$-3, $J_{(3,4)}$ = 2.76 Hz), 4.529 (1H, dd, H$_{dF}$-3, $J_{(2,3)}$= 10.38 Hz, $J_{(3,4)}$ = 2.82 Hz), 4.418 (1H, dd, H$_{[?]U}$-3, $J_{(2,3)}$ = 7.56 Hz,$J_{(3,4)}$ = 2.82 Hz), 4.363 (1H, m, H$_{rF}$-5, $J_{(5,6)}$ = 6.84 Hz), 4.273 (1H, m, H$_{dF}$-5, $J_{(5,6)}$ = 6.48 Hz), 4.268 (1H, m, H$_A$-6, $J_{(6,6')}$ = 10.68 Hz), 4.256 (1H, d, H$_{rU}$-5,$J_{(4,5)}$ = 7.44 Hz), 4.167 (1H, dd, H$_A$-3, $J_{(2,3)}$ = 8.70 Hz,$J_{(3,4)}$ = 2.16 Hz), 4.146 (1H, m, H$_A$-6', $J_{(5,6')}$ = 3.52 Hz), 4.071 (1H, dd, H$_{rU}$-4, $J_{(4,5)}$ = 7.44 Hz), 4.060 (1H, dd, H$_{rU}$-2, $J_{(1,2)}$= 3.96 Hz, $J_{(1',2)}$ = 6.78 Hz), 4.043 (1H, dd, H$_A$-2, $J_{(1,2)}$ = 8.52 Hz,$J_{(2,3)}$ = 8.70 Hz), 4.019 (1H, m, H$_A$-5, $J_{(5,6/6')}$ = 8.24 Hz, 3.52 Hz), 4.019 (1H, dd, H$_{rU}$-3), 3.886 (1H, dd, H$_{dF}$-2, $J_{(1,2)}$ = 3.78 Hz,$J_{(2,3)}$ = 10.34 Hz), 3.886 (1H, dd, H$_{rF}$-2, $J_{(2,3)}$ = 10.38 Hz), 3.822 (1H, dd, H$_{[?]U}$-2, $J_{(1,2)}$ = 8.64 Hz,$J_{(2,3)}$ = 7.56 Hz), 3.743 (1H, d, H$_{rU}$-1, $J_{(1,2)}$ = 3.96 Hz,$J_{(1,1')}$ = 11.82 Hz), 3.700 (1H, d, H$_{rU}$-1', $J_{(1',2)}$ = 6.78 Hz), 1.986 (3H, s, H$_A$-8), 1.240 (3H, d, H$_{rF}$-6,$J_{(5,6)}$ = 6.84 Hz), 1.231 (3H, d, H$_{dF}$-6, $J_{(5,6)}$ = 6.32 Hz);^{13}C NMR (200 MHz, D₂O) δ 180.08 (C$_{rU}$-6), 177.65 (C$_A$-7), 171.65 (C$_{[?]U}$-6), 149.51 (C$_{[?]U}$-5), 109.52 (C$_{[?]U}$-4), 105.82 (C$_{[?]U}$-1), 104.31 (C$_A$-1), 104.26 (C$_{rF}$-1), 101.04 (C$_{dF}$-1), 84.36 (C$_{rU}$-4), 82.44 (C$_{rU}$-3), 81.70 (C$_{rF}$-4), 81.72 (C$_{dF}$-4), 79.12 (C$_{[?]U}$-3),78.94 (C$_A$-4), 78.56 (C$_A$-3), 75.17 (C$_{rU}$-5), 74.64 (C$_A$-5), 72.90 (C$_{[?]U}$-2), 72.59 (C$_{rU}$-2), 70.64 (C$_A$-6), 69.55 (C$_{rF}$-5), 69.39 (C$_{rF}$-2), 69.23 (C$_{dF}$-5), 69.02 (C$_{dF}$-2), 65.28 (C$_{rU}$-1), 54.35 (C$_A$-2), 25.19 (C$_A$-8), 18.85 (C$_{rF}$-6), 18.56 (C$_{dF}$-6); HRMS (ESI-Q-TOF) m/z: [M-2Na]$^{2-}$ calculated for $C_{32}H_{43}O_{43}NS_6Na_6$729.4465; Found for 729.4412.

oHG-8, octasaccharide ,L-Φυς₃Σ₄Σ-α(1,3)-Λ-Δ^{4,5}ΓλςΑ-α(1,3)-Δ-ΓαλΝΑς₄Σ₆Σ-β(1,4)-[Λ-Φυς₃Σ₄Σ-α(1,]3)-Δ-ΓλςΑ-β(1,3)-Δ-ΓαλΝΑς₄Σ₆Σ-β(1,4)-[Λ-Φυς₃Σ₄Σ-(α1,] 3)-Δ-ΓλςΑ-oλ: ^{1}H NMR

(800 MHz, D$_2$O) δ5.686 (1H, d, H$_{[?]U}$-4), 5.286 (1H, d, H$_F$-1,J $_{(1,2)}$ = 3.72 Hz), 5.204 (1H, d, H$_{dF}$-1, J $_{(1,2)}$ = 3.78 Hz), 5.033 (1H, d, H$_{rF}$-1, J $_{(1,2)}$ = 3.84 Hz), 4.973 (1H, d, H$_F$-4, J $_{(3,4)}$ = 2.46 Hz), 4.912 (1H, m, H$_{dA}$-4), 4.846 (1H, d, H$_{[?]U}$-1, J $_{(1,2)}$ = 8.28 Hz), 4.831 (1H, d, H$_{dF}$-4, J $_{(3,4)}$ = 2.82 Hz), 4.837 (1H, d, H$_{rF}$-4, J $_{(3,4)}$ =3.06 Hz), 4.799 (1H, m, H$_F$-5, J $_{(5,6)}$ = 6.36 Hz), 4.622 (1H, d, H$_{rA}$-1), 4.545 (1H, d, H$_{rF}$-3, J $_{(3,4)}$ = 3.06 Hz), 4.530 (1H, d, H$_{dA}$-1, J $_{(1,2)}$ = 8.52 Hz), 4.530 (1H, d, H$_{dF}$-3, J $_{(3,4)}$ = 2.82 Hz), 4.458 (1H, d, H$_F$-3,J $_{(3,4)}$ = 2.46 Hz), 4.426 (1H, dd, H$_{[?]U}$-3, J $_{(2,3)}$ = 8.22 Hz,J $_{(3,4)}$ = 2.40 Hz), 4.426 (1H, d, H$_U$-1, J $_{(1,2)}$ = 8.16 Hz), 4.370 (1H, m, H$_{rF}$-5, J $_{(5,6)}$ = 6.84 Hz), 4.532 (1H, d, H$_{dA}$-6,J $_{(1,2)}$ = 8.16 Hz), 4.418 (1H, dd, H$_{dF}$-5, J $_{(1,2)}$ = 3.92 Hz,J $_{(2,3)}$ = 10.60 Hz), 4.321 (1H, m, H$_{rA}$-6, J $_{(6,6')}$ = 12.06 Hz), 4.129 (1H, m, H$_{rA}$-6', J $_{(6,6')}$ = 10.68 Hz), 4.090 (1H, dd, H$_{dA}$-2,J $_{(1,2)}$ = 8.52 Hz), 4.087 (1H, dd, H$_{dA}$-3), 4.066 (1H, m, H$_{rU}$-2, J $_{(1,2)}$ = 3.96 Hz), 4.012 (1H, m, H$_{rU}$-3), 4.012 (1H, m, H$_{rU}$-4), 4.022 (1H, m, H$_{rA}$-5, J $_{(5,6)}$ = 8.24 Hz), 4.019 (1H, m, H$_{dA}$-5), 3.976 (1H, m, H$_{rA}$-2, J $_{(1,2)}$ = 7.26 Hz), 3.981 (1H, m, H$_{rA}$-3), 3.954 (1H, d, H$_U$-4,J $_{(4,5)}$ = 9.42 Hz), 3.886 (1H, dd, H$_{rF}$-2, J $_{(1,2)}$ = 3.84 Hz,J $_{(2,3)}$ = 10.38 Hz), 3.886 (1H, dd, H$_{dF}$-2,J $_{(1,2)}$ = 3.78 Hz, J $_{(2,3)}$ = 10.38 Hz), 3.871 (1H, dd, H$_F$-2,J $_{(1,2)}$ = 3.72 Hz, J $_{(2,3)}$ = 10.50 Hz), 3.830 (1H, dd, H$_{[?]U}$-2, J $_{(1,2)}$ = 8.28 Hz,J $_{(2,3)}$ = 8.22 Hz), 3.733 (1H, d, H$_{rU}$-1,J $_{(1,1')}$ = 11.88 Hz, J $_{(1,2)}$= 3.96 Hz), 3.691 (1H, d, H$_{rU}$-1',J $_{(1',2)}$ = 6.84 Hz),3.632 (1H, dd, H$_U$-3, J $_{(2,3)}$ = 9.06 Hz,J $_{(3,4)}$ = 8.61 Hz), 3.641 (1H, d, H$_U$-5, J $_{(4,5)}$ = 9.42 Hz), 3.546 (1H, dd, H$_U$-2, J $_{(1,2)}$ = 8.16 Hz,J $_{(2,3)}$ =9.06 Hz), 1.997 (3H, s, H$_{dA}$-8), 1.992 (3H, s, H$_{rA}$-8), 1.343 (3H, d, H$_F$-6, J $_{(5,6)}$ = 6.36 Hz),1.241 (3H, d, H$_{rF}$-6, J $_{(5,6)}$ = 6.84 Hz), 1.231 (3H, d, H$_{dF}$-6, J $_{(5,6)}$ = 6.48 Hz); ^{13}C NMR (200 MHz, D$_2$O)δ 180.08 (C$_{rU}$-6), 177.88 (C$_U$-6), 177.74 (C$_{rA}$-7, C$_{dA}$-7), 171.65 (C$_{[?]U}$-6), 149.61 (C$_{[?]U}$-5), 109.44 (C$_{[?]U}$-4), 106.44 (C$_U$-1),105.81 (C$_{[?]U}$-1), 104.17 (C$_{rA}$-1), 104.17 (C$_{rF}$-1), 102.39 (C$_{dA}$-1), 101.97 (C$_F$-1), 100.94 (C$_{dF}$-1), 84.03 (C$_{rU}$-4), 82.33 (C$_{rU}$-3), 82.09 (C$_F$-4), 81.88 (C$_U$-3), 81.68 (C$_{rF}$-4), 81.69 (C$_{dF}$-4), 79.86 (C$_U$-5), 79.10 (C$_{[?]U}$-3), 78.98 (C$_{dA}$-4, C$_{rA}$-4), 78.55 (C$_{dA}$-3), 78.07 (C$_{rA}$-3), 78.07 (C$_F$-3), 77.91 (C$_{rF}$-3), 77.96 (C$_{dF}$-3), 77.91 (C$_U$-4), 76.24 (C$_U$-2), 75.03 (C$_{rA}$-5), 74.62 (C$_{rU}$-5, C$_{dA}$-5), 72.96 (C$_{[?]U}$-2), 72.59 (C$_{rU}$-2), 70.52 (C$_{rA}$-6), 69.99 (C$_{dA}$-6), 69.52 (C$_{rF}$-5), 69.36 (C$_{dF}$-5), 69.24 (C$_{rF}$-2), 69.10 (C$_{dF}$-2), 69.10 (C$_F$-2), 69.00 (C$_F$-5), 65.21 (C$_{rU}$-1), 54.26 (C$_{rA}$-2), 54.26 (C$_{dA}$-2), 25.28 (C$_{rA}$-8), 25.26 (C$_{dA}$-8), 18.84 (C$_{rF}$-6), 18.78 (C$_F$-6), 18.56 (C$_{dF}$-6); HRMS (ESI-Q-TOF) m/z: [M-3Na]$^{3-}$ calculated for C$_{52}$H$_{69}$N$_2$Na$_{10}$O$_{70}$S$_{10}$$^{3-}$796.9367; Found for 796.9317.

oHG-11, hendecasaccharide,L-**Φυς$_{3\Sigma 4\Sigma}$-α(1,3)-**$Λ$-**Δ^{4,5}Γλς A-α(1,3)-{Δ-ΓαλNAς$_{4\Sigma 6\Sigma}$-β(1,4)-[$Λ$-Φυς$_{3\Sigma 4\Sigma}$-α(1,]3)-Δ-Γλς A-β(1,3)-}$_2$-Δ-ΓαλNAς$_{4\Sigma 6\Sigma}$-β(1,4)-[$Λ$-Φυς$_{3\Sigma 4\Sigma}$-(α1,]3)-Δ-Γλς A-ολ:**^{1}H NMR (800 MHz, D$_2$O) δ 5.686 (1H, d, H$_{[?]U}$-4), 5.277 (1H, d, H$_F$-1,J $_{(1,2)}$ = 4.88 Hz), 5.204 (1H, d, H$_{dF}$-1, J $_{(1,2)}$ = 3.78 Hz), 5.033 (1H, d, H$_{rF}$-1, J $_{(1,2)}$ = 3.84 Hz), 4.957 (1H, d, H$_F$-4, J $_{(3,4)}$ = 4.88 Hz), 4.905 (1H, m, H$_{dA}$-4), 4.842 (1H, d, H$_{[?]U}$-1, J $_{(1,2)}$ = 8.30 Hz), 4.832 (1H, d, H$_{rF}$-4,J $_{(3,4)}$=3.06 Hz), 4.826 (1H, d, H$_{dF}$-4,J $_{(3,4)}$ = 2.82 Hz), 4.783 (1H, m, H$_F$-5, J $_{(5,6)}$ = 6.36 Hz), 4.752(1H, m,H$_{rA}$-4), 4.750 (1H, H$_A$-4), 4.620 (1H, d, H$_{rA}$-1, J $_{(1,2)}$ =7.68 Hz), 4.544 (1H, d, H$_{rF}$-3, J $_{(3,4)}$= 3.06 Hz), 4.530 (1H, d, H$_{dF}$-3, J $_{(3,4)}$ = 2.82 Hz), 4.515 (1H, d, H$_{dA/A}$-1, J $_{(1,2)}$ = 8.52 Hz), 4.498 (1H, d, H$_A$-1, J $_{(1,2)}$ = 8.52 Hz), 4.449 (1H, d, H$_F$-3,J $_{(3,4)}$ = 2.64 Hz), 4.425 (1H, dd, H$_{[?]U}$-3, J $_{(2,3)}$ = 8.16 Hz,J $_{(3,4)}$ = 2.72 Hz), 4.425 (1H, d, H$_U$-1, J $_{(1,2)}$ = 8.16 Hz), 4.354 (1H, m, H$_{rF}$-5, J $_{(5,6)}$ = 6.64 Hz), 4.315 (1H, d, H$_{dA}$-6, J $_{(6,6')}$ =12.06 Hz), 4.283 (1H, dd, H$_{dF}$-5, J $_{(5,6)}$ = 6.48 Hz), 4.200 (1H, m, H$_{rA}$-6, J $_{(6,6')}$= 10.68 Hz), 4.199 (1H, d, H$_A$-6,J $_{(6,6')}$ = 12.06 Hz), 4.117 (1H, m, H$_{rA}$-6',J $_{(6,6')}$ = 10.68 Hz), 4.105 (1H, m, H$_{dA/A}$-6', J $_{(6,6')}$ = 12.06 Hz), 4.094 (1H, dd, H$_{dA}$-2, J $_{(1,2)}$ = 8.52 Hz), 4.082 (1H, dd, H$_{dA}$-3), 4.064 (1H, m, H$_{rU}$-2, J $_{(1,2)}$ = 4.00 Hz), 4.013 (1H, m, H$_A$-2, J $_{(1,2)}$ = 8.52 Hz), 4.009 (1H, m, H$_{dA}$-5,J $_{(5,6')}$ = 5.34 Hz), 4.006 (1H, m, H$_{rU}$-3), 4.006 (1H, m, H$_{rU}$-4), 4.008 (1H, m, H$_{rA}$-5), 3.993 (1H, m, H$_{rA}$-2,J $_{(1,2)}$ = 7.68 Hz), 3.980 (1H, m, H$_{rA}$-3), 3.940 (1H, m, H$_A$-5,J $_{(5,6')}$ = 5.34 Hz), 3.936 (1H, d, H$_U$-4, J $_{(4,5)}$ = 9.42 Hz), 3.895 (1H, m, H$_A$-3), 3.884 (1H, dd, H$_{rF}$-2,J $_{(1,2)}$ = 4.08 Hz, J $_{(2,3)}$ = 10.72 Hz), 3.884 (1H, dd, H$_{dF}$-2,J $_{(1,2)}$ = 4.00 Hz, J $_{(2,3)}$ = 10.38 Hz), 3.862 (1H, dd, H$_F$-2,J $_{(1,2)}$ = 4.88 Hz, J $_{(2,3)}$ = 10.50 Hz), 3.825 (1H, dd, H$_{[?]U}$-2,J $_{(1,2)}$ = 8.30 Hz, J $_{(2,3)}$ = 8.16 Hz), 3.729 (1H, d, H$_{rU}$-1, J $_{(1,2)}$ = 11.92 Hz), 3.691 (1H, d, H$_{rU}$-1', J $_{(1',2)}$ = 6.72 Hz), 3.632 (1H, d, H$_U$-5, J $_{(4,5)}$ = 9.42 Hz), 3.616 (1H, dd, H$_U$-3,J $_{(2,3)}$ = 8.24 Hz, J $_{(3,4)}$ = 8.61 Hz), 3.542 (1H, dd, H$_U$-2,J $_{(1,2)}$ = 8.16 Hz, J $_{(2,3)}$ =8.24 Hz), 1.998 (3H, s, H$_{rA/A}$-8), 1.988 (3H, s, H$_{dA}$-8), 1.328 (3H, d, H$_F$-6,J $_{(5,6)}$ = 6.36 Hz),1.239 (3H, d, H$_{rF}$-6, J $_{(5,6)}$ = 6.64 Hz), 1.230 (3H, d, H$_{dF}$-6, J $_{(5,6)}$ = 6.48 Hz);^{13}C NMR (200 MHz, D$_2$O) δ 180.08 (C$_{rU}$-6), 177.72 (C$_{(r/d)A}$-7), 177.72 (C$_U$-6), 171.60 (C$_{[?]U}$-6), 149.64 (C$_{[?]U}$-5), 109.38 (C$_{[?]U}$-4), 106.50 (C$_U$-1),105.78 (C$_{[?]U}$-1), 104.16 (C$_{rA}$-1), 104.16 (C$_{rF}$-1), 102.33 (C$_{dA/A}$-1), 101.98 (C$_F$-1), 100.99 (C$_{dF}$-1), 84.03 (C$_{rU}$-4), 82.14 (C$_{rU}$-3), 82.11 (C$_U$-3), 81.95 (C$_F$-4), 81.70 (C$_{r/dF}$-4), 79.85 (C$_U$-5),

79.11 (C$_{[?]U}$-3), 78.99 (C$_{(d/r) A}$-4), 78.78 (C$_{(r/d)A}$-3), 78.11 (C$_F$-3), 77.98 (C$_U$-4), 77.94 (C$_{r/dF}$-3), 76.28 (C$_U$-2), 75.04 (C$_{rA}$-5), 74.68 (C$_{rU}$-5), 74.61(C$_{(d)A}$-5), 73.00 (C$_{(r/d)U}$-2), 70.53 (C$_{rA}$-6), 69.85 (C$_{dA}$-6), 70.03 (C$_A$-6), 69.55 (C$_{rF}$-5),69.24 (C$_{dF}$-5), 69.39 (C$_{rF}$-2), 69.39 (C$_{dF}$-2), 69.13 (C$_F$-2), 69.02 (C$_F$-5), 65.25 (C$_{rU}$-1), 54.23 (C$_{(r/d)A}$-2), 25.29 (C$_{(r/d)A}$-8), 18.83 (C$_F$-6), 18.79 (C$_{rF}$-6), 18.56 (C$_{dF}$-6); HRMS (ESI-Q-TOF) m/z: [M-3Na]$^{3-}$ calculated for C$_{72}$H$_{95}$N$_3$Na$_{15}$O$_{97}$S$_{14}$$^{3-}$1115.2388; Found for 1115.2299.

οΗΓ-14, τετραδεςασαςςηαριδε,Λ-Φυς$_{3Σ4Σ}$-α(1,3)-Λ-Δ^{4,5}ΓλςΑ-α(1,3)-{Δ-ΓαλΝΑς$_{4Σ6Σ}$-β(1,4)-[Λ-Φυς$_{3Σ4Σ}$-α(1,]3)-Δ-ΓλςΑ-β(1,3)-}$_3$-Δ-ΓαλΝΑς$_{4Σ6Σ}$-β(1,4)-[Λ-Φυς$_{3Σ4Σ}$-(α1,]3)-Δ-ΓλςΑ-ολ:^{1}H NMR (800 MHz, D$_2$O) δ 5.681 (1H, d, H$_{[?]U}$-4), 5.279 (1H, d, H$_F$-1,$J$$_{(1,2)}$ = 4.08 Hz), 5.200 (1H, d, H$_{dF}$-1, $J$$_{(1,2)}$ = 4.00 Hz), 5.034 (1H, d, H$_{rF}$-1, $J$$_{(1,2)}$ = 4.08 Hz), 4.956 (1H, d, H$_F$-4, $J$$_{(3,4)}$ = 2.64 Hz), 4.905 (1H, m, H$_{dA}$-4), 4.845 (1H, d, H$_{[?]U}$-1, $J$$_{(1,2)}$ = 8.30 Hz), 4.832 (1H, d, H$_{rF}$-4, $J$$_{(3,4)}$ =4.30 Hz), 4.826 (1H, d, H$_{dF}$-4, $J$$_{(3,4)}$ = 2.82 Hz), 4.782 (1H, m, H$_F$-5,$J$$_{(5,6)}$ = 6.36 Hz), 4.753 (1H, m, H$_{rA}$-4), 4.754 (1H, H$_A$-4), 4.621 (1H, d, H$_{rA}$-1, $J$$_{(1,2)}$ =6.80 Hz), 4.545 (1H, d, H$_{rF}$-3, $J$$_{(3,4)}$ = 4.30 Hz), 4.528 (1H, d, H$_{dF}$-3, $J$$_{(3,4)}$ = 2.82 Hz), 4.500 (1H, d, H$_{dA/A}$-1,$J$$_{(1,2)}$ = 8.52 Hz), 4.492 (1H, d, H$_A$-1, $J$$_{(1,2)}$ = 8.52 Hz), 4.443 (1H, d, H$_F$-3, $J$$_{(3,4)}$ = 2.64 Hz), 4.430 (1H, dd, H$_{[?]U}$-3, $J$$_{(2,3)}$ = 8.16 Hz, $J$$_{(3,4)}$ = 2.72 Hz), 4.415 (1H, d, H$_U$-1, $J$$_{(1,2)}$ = 8.16 Hz), 4.355 (1H, m, H$_{rF}$-5, $J$$_{(5,6)}$ = 6.56 Hz), 4.313 (1H, d, H$_{dA}$-6, $J$$_{(6,6')}$=12.06 Hz), 4.282 (1H, dd, H$_{dF}$-5,$J$$_{(5,6)}$ = 6.48 Hz), 4.226 (1H, m, H$_{rA}$-6, $J$$_{(6,6')}$ = 10.68 Hz), 4.280 (1H, d, H$_A$-6, $J$$_{(6,6')}$ = 12.06 Hz), 4.093 (1H, m, H$_{rA}$-6'), 4.189 (1H, m, H$_{dA/A}$-6', $J$$_{(6,6')}$ = 12.06 Hz), 4.078 (1H, dd, H$_{dA}$-2, $J$$_{(1,2)}$ = 8.52 Hz), 4.076 (1H, dd, H$_{dA}$-3), 4.073 (1H, m, H$_{rU}$-2, $J$$_{(1,2)}$ = 3.76 Hz), 3.977 (1H, m, H$_A$-2, $J$$_{(1,2)}$ = 8.52 Hz), 4.009 (1H, m, H$_{dA}$-5, $J$$_{(5,6')}$ = 5.34 Hz), 4.009 (1H, m, H$_{rU}$-3), 4.009 (1H, m, H$_{rU}$-4), 4.009 (1H, m, H$_{rA}$-5), 3.996 (1H, m, H$_{rA}$-2, $J$$_{(1,2)}$ = 6.80 Hz), 3.982 (1H, m, H$_{rA}$-3), 3.934 (1H, m, H$_A$-5, $J$$_{(5,6')}$ = 5.34 Hz), 3.935 (1H, d, H$_U$-4, $J$$_{(4,5)}$ = 9.42 Hz), 3.866 (1H, m, H$_A$-3), 3.886 (1H, dd, H$_{rF}$-2, $J$$_{(1,2)}$ = 4.08 Hz,$J$$_{(2,3)}$ = 10.88 Hz), 3.885 (1H, dd, H$_{dF}$-2, $J$$_{(1,2)}$ = 4.00 Hz,$J$$_{(2,3)}$ = 10.38 Hz), 3.873 (1H, dd, H$_F$-2, $J$$_{(1,2)}$ = 4.08 Hz, $J$$_{(2,3)}$ = 10.50 Hz), 3.826 (1H, dd, H$_{[?]U}$-2, $J$$_{(1,2)}$ = 8.30 Hz,$J$$_{(2,3)}$ = 8.16 Hz), 3.729 (1H, d, H$_{rU}$-1, $J$$_{(1,2)}$ = 11.84 Hz), 3.691 (1H, d, H$_{rU}$-1',$J$$_{(1,1')}$ = 11.84 Hz, $J$$_{(1',2)}$ = 6.80 Hz), 3.632 (1H, d, H$_U$-5, $J$$_{(4,5)}$ = 9.42 Hz), 3.620 (1H, dd, H$_U$-3, $J$$_{(2,3)}$ = 8.00 Hz,$J$$_{(3,4)}$ = 8.64 Hz), 3.540 (1H, dd, H$_U$-2, $J$$_{(1,2)}$ = 8.16 Hz,$J$$_{(2,3)}$ =8.00 Hz), 1.998 (3H, s, H$_{rA/A}$-8), 1.988 (3H, s, H$_{dA}$-8), 1.323 (3H, d, H$_F$-6, $J$$_{(5,6)}$ = 6.36 Hz),1.239 (3H, d, H$_{rF}$-6, $J$$_{(5,6)}$ = 6.56 Hz), 1.230 (3H, d, H$_{dF}$-6,$J$$_{(5,6)}$ = 6.48 Hz); ^{13}C NMR (200 MHz, D$_2$O) δ 180.06 (C$_{rU}$-6), 177.82 (C$_{(r/d)A}$-7), 177.72 (C$_U$-6), 171.60 (C$_{[?]U}$-6), 149.65 (C$_{[?]U}$-5), 109.38 (C$_{[?]U}$-4), 106.51 (C$_U$-1),105.78 (C$_{[?]U}$-1), 104.09 (C$_{rA}$-1), 104.09 (C$_{rF}$-1), 102.33 (C$_{dA/A}$-1), 101.97 (C$_F$-1), 101.00 (C$_{dF}$-1), 84.03 (C$_{rU}$-4), 82.39 (C$_{rU}$-3), 82.11 (C$_U$-3), 81.98 (C$_F$-4), 81.73 (C$_{r/dF}$-4), 79.75 (C$_U$-5), 79.23 (C$_{[?]U}$-3), 79.15 (C$_{(d/r) A}$-4), 78.54 (C$_{(r/d)A}$-3), 78.12 (C$_F$-3), 77.98 (C$_U$-4), 77.98 (C$_{r/dF}$-3), 76.29 (C$_U$-2), 75.05 (C$_{rA}$-5), 74.69 (C$_{rU}$-5), 74.61(C$_{(d)A}$-5), 73.01 (C$_{r/dU}$-2), 70.53 (C$_{rA}$-6), 69.89 (C$_{dA}$-6), 70.03 (C$_A$-6), 69.55 (C$_{rF}$-5),69.24 (C$_{dF}$-5), 69.40 (C$_{rF}$-2), 69.40 (C$_{dF}$-2), 69.13 (C$_F$-2), 69.03 (C$_F$-5), 65.26 (C$_{rU}$-1), 54.23 (C$_{(r/d)A}$-2), 25.29 (C$_{(r/d)A}$-8), 18.80 (C$_F$-6), 18.83 (C$_{rF}$-6), 18.56 (C$_{dF}$-6); HRMS (ESI-Q-TOF) m/z: [M-6Na]$^{6-}$ calculated for C$_{92}$H$_{121}$N$_4$Na$_{17}$O$_{124}$S$_{18}$$^{6-}$705.2759; Found for 705.2753.

οHG-17, heptadecasaccharide,L-Φυς$_{3Σ4Σ}$-α(1,3)-Λ-Δ^{4,5}ΓλςΑ-α(1,3)-{Δ-ΓαλΝΑς$_{4Σ6Σ}$-β(1,4)-[Λ-Φυς$_{3Σ4Σ}$-α(1,]3)-Δ-ΓλςΑ-β(1,3)-}$_4$-Δ-ΓαλΝΑς$_{4Σ6Σ}$-β(1,4)-[Λ-Φυς$_{3Σ4Σ}$-(α1,]3)-Δ-ΓλςΑ-ολ:^{1}H NMR (800 MHz, D$_2$O) δ 5.681 (1H, d, H$_{[?]U}$-4), 5.279 (1H, d, H$_F$-1,$J$$_{(1,2)}$ = 4.08 Hz), 5.200 (1H, d, H$_{dF}$-1, $J$$_{(1,2)}$ = 4.00 Hz), 5.034 (1H, d, H$_{rF}$-1, $J$$_{(1,2)}$ = 4.08 Hz), 4.956 (1H, d, H$_F$-4, $J$$_{(3,4)}$ = 2.64 Hz), 4.905 (1H, m, H$_{dA}$-4), 4.845 (1H, d, H$_{[?]U}$-1, $J$$_{(1,2)}$ = 8.30 Hz), 4.832 (1H, d, H$_{rF}$-4, $J$$_{(3,4)}$ =4.30 Hz), 4.826 (1H, d, H$_{dF}$-4, $J$$_{(3,4)}$ = 2.82 Hz), 4.782 (1H, m, H$_F$-5,$J$$_{(5,6)}$ = 6.36 Hz), 4.753 (1H, m, H$_{rA}$-4), 4.754 (1H, H$_A$-4), 4.621 (1H, d, H$_{rA}$-1, $J$$_{(1,2)}$ =6.80 Hz), 4.545 (1H, d, H$_{rF}$-3, $J$$_{(3,4)}$ = 4.30 Hz), 4.528 (1H, d, H$_{dF}$-3, $J$$_{(3,4)}$ = 2.82 Hz), 4.500 (1H, d, H$_{dA/A}$-1,$J$$_{(1,2)}$ = 8.52 Hz), 4.492 (1H, d, H$_A$-1, $J$$_{(1,2)}$ = 8.52 Hz), 4.443 (1H, d, H$_F$-3, $J$$_{(3,4)}$ = 2.64 Hz), 4.430 (1H, dd, H$_{[?]U}$-3, $J$$_{(2,3)}$ = 8.16 Hz, $J$$_{(3,4)}$ = 2.72 Hz), 4.415 (1H, d, H$_U$-1, $J$$_{(1,2)}$ = 8.16 Hz), 4.355 (1H, m, H$_{rF}$-5, $J$$_{(5,6)}$ = 6.56 Hz), 4.313 (1H, d, H$_{dA}$-6, $J$$_{(6,6')}$=12.06 Hz), 4.282 (1H, dd, H$_{dF}$-5,$J$$_{(5,6)}$ = 6.48 Hz), 4.226 (1H, m, H$_{rA}$-6, $J$$_{(6,6')}$ = 10.68 Hz), 4.280 (1H, d, H$_A$-6, $J$$_{(6,6')}$ = 12.06 Hz), 4.093 (1H, m, H$_{rA}$-6'), 4.189 (1H, m, H$_{dA/A}$-6', $J$$_{(6,6')}$ = 12.06 Hz), 4.078 (1H, dd, H$_{dA}$-2, $J$$_{(1,2)}$ = 8.52 Hz), 4.076 (1H, dd, H$_{dA}$-3), 4.073 (1H, m, H$_{rU}$-2, $J$$_{(1,2)}$ = 3.76 Hz), 3.977 (1H, m, H$_A$-2, $J$$_{(1,2)}$ = 8.52 Hz), 4.009 (1H, m, H$_{dA}$-5, $J$$_{(5,6')}$ = 5.34 Hz), 4.009 (1H, m, H$_{rU}$-3), 4.009 (1H, m,

H_{rU}-4), 4.009 (1H, m, H_{rA}-5), 3.996 (1H, m, H_{rA}-2, $J_{(1,2)}$ = 6.80 Hz), 3.982 (1H, m, H_{rA}-3), 3.934 (1H, m, H_A-5, $J_{(5,6')}$ = 5.34 Hz), 3.935 (1H, d, H_U-4, $J_{(4,5)}$ = 9.42 Hz), 3.866 (1H, m, H_A-3), 3.886 (1H, dd, H_{rF}-2, $J_{(1,2)}$ = 4.08 Hz, $J_{(2,3)}$ = 10.88 Hz), 3.885 (1H, dd, H_{dF}-2, $J_{(1,2)}$ = 4.00 Hz, $J_{(2,3)}$ = 10.38 Hz), 3.873 (1H, dd, H_F-2, $J_{(1,2)}$ = 4.08 Hz, $J_{(2,3)}$ = 10.50 Hz), 3.826 (1H, dd, $H_{[?]U}$-2, $J_{(1,2)}$ = 8.30 Hz, $J_{(2,3)}$ = 8.16 Hz), 3.729 (1H, d, H_{rU}-1, $J_{(1,2)}$ = 11.84 Hz), 3.691 (1H, d, H_{rU}-1', $J_{(1,1')}$ = 11.84 Hz, $J_{(1',2)}$ = 6.80 Hz), 3.632 (1H, d, H_U-5, $J_{(4,5)}$ = 9.42 Hz), 3.620 (1H, dd, H_U-3, $J_{(2,3)}$ = 8.00 Hz, $J_{(3,4)}$ = 8.64 Hz), 3.540 (1H, dd, H_U-2, $J_{(1,2)}$ = 8.16 Hz, $J_{(2,3)}$ = 8.00 Hz), 1.998 (3H, s, $H_{rA/A}$-8), 1.988 (3H, s, H_{dA}-8), 1.323 (3H, d, H_F-6, $J_{(5,6)}$ = 6.36 Hz), 1.239 (3H, d, H_{rF}-6, $J_{(5,6)}$ = 6.56 Hz), 1.230 (3H, d, H_{dF}-6, $J_{(5,6)}$ = 6.48 Hz); ^{13}C NMR (200 MHz, D_2O) δ 180.08 (C_{rU}-6), 177.82 ($C_{(r/d)A}$-7), 177.72 (C_U-6), 171.60 ($C_{[?]U}$-6), 149.64 ($C_{[?]U}$-5), 109.38 ($C_{[?]U}$-4), 106.51 (C_U-1), 105.78 ($C_{[?]U}$-1), 104.16 (C_{rA}-1), 104.16 (C_{rF}-1), 102.33 ($C_{dA/A}$-1), 101.98 (C_F-1), 100.99 (C_{dF}-1), 84.03 (C_{rU}-4), 82.14 (C_{rU}-3), 82.11 (C_U-3), 81.95 (C_F-4), 81.73 ($C_{r/dF}$-4), 79.85 (C_U-5), 79.11 ($C_{[?]U}$-3), 78.99 ($C_{(d/r) A}$-4), 78.78 ($C_{(r/d)A}$-3), 78.11 (C_F-3), 77.98 (C_U-4), 77.91 ($C_{r/dF}$-3), 76.28 (C_U-2), 75.04 (C_{rA}-5), 74.68 (C_{rU}-5), 74.61($C_{(d)A}$-5), 72.77 (C_{rU}-2), 73.00 (C_{dU}-2), 70.53 (C_{rA}-6), 69.85 (C_{dA}-6), 70.03 (C_A-6), 69.55 (C_{rF}-5), 69.24 (C_{dF}-5), 69.39 (C_{rF}-2), 69.39 (C_{dF}-2), 69.13 (C_F-2), 69.02 (C_F-5), 65.25 (C_{rU}-1), 54.23 ($C_{(r/d)A}$-2), 25.29 ($C_{(r/d)A}$-8), 18.83 (C_F-6), 18.79 (C_{rF}-6), 18.56 (C_{dF}-6); HRMS (ESI-Q-TOF) m/z: [M-4Na]$^{4-}$ calculated for $C_{112}H_{147}N_5Na_{24}O_{151}S_{22}$$^{4-}$1308.8784; Found for 1308.3858.

dHG-5, L-Φυς$_{3Σ4Σ}$-α(1,3)-$Λ$-$Δ^{4,5}$ΓλςA-α(1,3)-{$Δ$-ΓαλNAς$_{4Σ6Σ}$-β(1,4)-[$Λ$-Φυς$_{3Σ4Σ}$-α(1,]3)-$Δ$-ΓλςA-β(1,3)-}$_ν$-$Δ$-ΓαλNAς$_{4Σ6Σ}$-β(1,4)-[$Λ$-Φυς$_{3Σ4Σ}$-(α1,]3)-$Δ$-ΓλςA-ολ, n=4: ^{1}H NMR (800 MHz, D_2O) δ 5.685 (1H, d, $H_{[?]U}$-4), 5.283 (1H, d, H_F-1, $J_{(1,2)}$ = 2.72 Hz), 5.201 (1H, d, H_{dF}-1, $J_{(1,2)}$ = 3.36 Hz), 5.030 (1H, d, H_{rF}-1, $J_{(1,2)}$ = 3.24 Hz), 4.970 (1H, d, H_F-4), 4.908 (1H, m, H_{dA}-4), 4.843 (1H, d, $H_{[?]U}$-1, $J_{(1,2)}$ = 8.80 Hz), 4.834 (1H, d, H_{rF}-4, $J_{(3,4)}$ = 2.40 Hz), 4.830 (1H, d, H_{dF}-4, $J_{(3,4)}$ = 2.40 Hz), 4.786 (1H, m, H_F-5, $J_{(5,6)}$ = 6.00 Hz), 4.763 (1H, m, H_{rA}-4), 4.751 (1H, H_A-4), 4.620 (1H, d, H_{rA}-1, $J_{(1,2)}$ = 6.88 Hz), 4.544 (1H, d, H_{rF}-3, $J_{(3,4)}$ = 2.40 Hz), 4.530 (1H, d, H_{dF}-3, $J_{(3,4)}$ = 2.40 Hz), 4.506 (1H, d, H_{dA}-1, $J_{(1,2)}$ = 8.80 Hz), 4.485 (1H, d, H_A-1, $J_{(1,2)}$ = 8.80 Hz), 4.437 (1H, d, H_F-3), 4.423 (1H, dd, $H_{[?]U}$-3, $J_{(2,3)}$ = 8.16 Hz), 4.398 (1H, d, H_U-1, $J_{(1,2)}$ = 8.80 Hz), 4.368 (1H, m, H_{rF}-5, $J_{(5,6)}$ = 6.48 Hz), 4.326 (1H, d, H_{dA}-6), 4.287 (1H, d, H_A-6), 4.284 (1H, dd, H_{dF}-5, $J_{(5,6)}$ = 6.96 Hz), 4.239 (1H, m, H_{rA}-6), 4.221 (1H, m, H_{dA}-6'), 4.183 (1H, m, H_A-6'), 4.104 (1H, m, H_{rA}-6'), 4.091 (1H, dd, H_{dA}-3), 4.081 (1H, dd, H_{dA}-2, $J_{(1,2)}$ = 8.80 Hz), 4.075 (1H, m, H_{rU}-2, $J_{(1,2)}$ = 3.36 Hz), 4.021 (1H, m, H_A-2, $J_{(1,2)}$ = 8.80 Hz), 4.012 (1H, m, H_{rA}-5), 4.010 (1H, m, H_{rU}-3), 4.007 (1H, m, H_{dA}-5), 4.005 (1H, m, H_{rU}-4), 3.993 (1H, m, H_{rA}-3), 3.974 (1H, m, H_{rA}-2, $J_{(1,2)}$ = 6.88 Hz), 3.935 (1H, d, H_U-4, $J_{(4,5)}$ = 8.00 Hz), 3.932 (1H, m, H_A-5), 3.895 (1H, m, H_A-3), 3.885 (1H, dd, H_{rF}-2, $J_{(1,2)}$ = 3.24 Hz, $J_{(2,3)}$ = 10.88 Hz), 3.885 (1H, dd, H_{dF}-2, $J_{(1,2)}$ = 3.36 Hz, $J_{(2,3)}$ = 10.88 Hz), 3.872 (1H, dd, H_F-2, $J_{(1,2)}$ = 2.72 Hz, $J_{(2,3)}$ = 9.04 Hz), 3.827 (1H, dd, $H_{[?]U}$-2, $J_{(1,2)}$ = 8.80 Hz, $J_{(2,3)}$ = 8.12 Hz), 3.729 (1H, d, H_{rU}-1, $J_{(1,2)}$ = 11.68 Hz), 3.692 (1H, d, H_{rU}-1', $J_{(1,1')}$ = 11.68 Hz, $J_{(1',2)}$ = 6.72 Hz), 3.63 (1H, d, H_U-5, $J_{(4,5)}$ = 8.00 Hz), 3.620 (1H, dd, H_U-3, $J_{(2,3)}$ = 8.56 Hz), 3.538 (1H, dd, H_U-2, $J_{(1,2)}$ = 8.80 Hz, $J_{(2,3)}$ = 8.56 Hz), 1.998 (3H, s, $H_{rA/A}$-8), 1.988 (3H, s, H_{dA}-8), 1.341 (3H, d, H_F-6, $J_{(5,6)}$ = 6.00 Hz), 1.239 (3H, d, H_{rF}-6, $J_{(5,6)}$ = 6.48 Hz), 1.230 (3H, d, H_{dF}-6, $J_{(5,6)}$ = 6.96 Hz); ^{13}C NMR (200 MHz, D_2O) δ 180.08 (C_{rU}-6), 177.90 ($C_{(r/d)A}$-7), 177.74 (C_U-6), 171.63 ($C_{[?]U}$-6), 149.62 ($C_{[?]U}$-5), 109.43 ($C_{[?]U}$-4), 106.55 (C_U-1), 105.81 ($C_{[?]U}$-1), 104.16 (C_{rA}-1), 104.16 (C_{rF}-1), 102.34 (C_A-1), 102.29 (C_{dA}-1), 101.93 (C_F-1), 100.95 (C_{dF}-1), 82.33 (C_{rU}-4), 82.32 (C_{rU}-3), 82.09 (C_U-3), 82.08 (C_F-4), 81.70 ($C_{r/dF}$-4), 79.65 (C_U-5), 79.20 ($C_{[?]U}$-3), 79.11 ($C_{(r)A}$-3), 78.98 (C_{rA}-3), 78.90 ($C_{(d/r) A}$-4), 78.56 (C_{dA}-3), 78.07 (C_F-3), 77.96 (C_U-4), 77.92 ($C_{r/dF}$-3), 76.36 (C_U-2), 75.03 (C_{rA}-5), 74.62 (C_{rU}-5), 74.62(C_A-5), 74.55(C_{dA}-5), 72.97 (C_{dU}-2), 72.61 (C_{rU}-2), 70.52 (C_{rA}-6), 70.03 (C_{dA}-6), 69.88 (C_A-6), 69.52 (C_{rF}-5), 69.24 (C_{dF}-5), 69.36 (C_{rF}-2), 69.36 (C_{dF}-2), 69.24 (C_{dF}-5), 69.09 (C_F-2), 69.00 (C_F-5), 65.21 (C_{rU}-1), 54.22 ($C_{(r/d)A}$-2), 25.26 ($C_{(r/d)A}$-8), 18.77 (C_F-6), 18.84 (C_{rF}-6), 18.56 (C_{dF}-6); HRMS (ESI-Q-TOF) m/z: [M-4Na]$^{4-}$ calculated for $C_{112}H_{147}N_5Na_{24}O_{151}S_{22}$$^{4-}$1308.8784; Found for 1308.3858.

Inhibition for iXase. Inhibition of compounds for iXase was determined using the Biophen f.VIII:C kits (containing R1, R2, and R3 solutions) by a Bio-Tek Microplate Reader (ELx 808, USA) as previously described (Wu, Wen *et al.* , 2015). Briefly, 30 μLeach compound solution at different concentrations (Tris-HCl solution as control), 30 μL f.VIII (2 IU/mL), and 30 μL R2 solution (containing 60 nM f.IXa, human

9

thrombin, phosphatidylcholine/phosphatidylserine and Ca^{2+}) were mixed and incubated at 37°C for 2 min. Then, 30 μL R1 solution (containing 50 nM f.X and thrombin inhibitor) was added. After incubation for 1 min at 37°C, the residual FXa activity was measured by the addition of 30 μL R3 solution (FXa chromogenic substrate SXa-11). Assays were conducted at 37°C in 96-well plates, compounds were dissolved in Tris buffer (0.02 M Tris/HCl, pH 7.4). Hydrolysis of the substrate resulted in the release of p-nitroaniline, the optical density (OD_{405nm}) was measured at 405nm (kinetic method, read per 30 s for 5 min). The change rate of absorbance at 405nm (ΔOD_{405nm}/min) in the presence of the test compound was normalized to that of the control to culculate the relative activity. The compound concentrations - relative activity were plotted and fitted by the following equation using the Origin 8.0 software (OriginLab, USA): $B = [IC_{50}]^n/\{[IC_{50}]^n+[I]^n\}$, where B represented the relative activity, [I] represented the compound concentrations, n represented the pseudo-Hill coefficient and IC_{50} represented the concentrations of compounds that inhibited 50% protease activity. Assays were carried out in duplicate.

Solution competition BLI study. The activities of compounds to compete with the immobilized oHG-11 for binding to f.IXa were performed on an Octet Red 96 instrument (Fortebio, USA). oHG-11 was biotinylated by reacting with amine-PEG3-biotin, then biotinylated oHG-11 was immobilized onto SA biosensors as previously described (B. Li, Suwan *et al.* , 2009; Xiao, Zhao *et al.* , 2019).

The kinetic assay of f.IXa binding to immobilized oHG-11 was conducted as below (Xiao, Lian *et al.* , 2016; Xiao, Zhao *et al.* , 2019). Firstly, baseline was equilibrated for 300 s with running buffer (0.15 M NaCl, 20 mM HEPES, pH 7.4, 2 mM $CaCl_2$, 0.05% Tween 20 and 0.1% BSA). Then, f.IXa with gradient concentrations associated with immobilized oHG-11 for 600 s. Next, the dissociation was conducted in running buffer for 900 s. Finally, the sensors were regenerated by regeneration buffer (2 M NaCl, 20 mM HEPES, pH 7.4, 2 mM $CaCl_2$, 0.05% Tween 20 and 0.1% BSA) for 10 s (repeated for 5 times). Assays were conducted once at 30°C in black 96-well flat bottom plates with agitation set to 1,000 revolutions per minute and final solution volume of 200 μL. Systematic baseline drift was corrected by subtracting the shift recorded by a sensor loaded with ligand but associated with no analyte. BLI kinetic data were analyzed by the Octet software version 7.0 and the binding curves were globally fitted with a 2:1 model (Figueralosada & Lograsso, 2012).

The relative ability of compounds binding to f.IXa was determined by competition binding assay (Xiao, Lian *et al.* , 2016; Xiao, Zhao *et al.* , 2019). f.IXa (100 nM) were pre-incubated with compounds with gradient concentrations prior to interaction with immobilized oHG-11. The competition binding assay including baseline equilibrium, association, dissociation and regeneration processes were performed as above. The relative response (f.IXa binding response with compound/ f.IXa binding response without compound) were calculated, and compound concentrations - relative response were fitted by the following equation with Origin 8.0 software (OriginLab, USA): $B = (IC_{50})^n / [(IC_{50})^n + [I]^n]$, where meaning of B, I, n and IC_{50} were the same as described above (John P Sheehan, Kobbervig *et al.* , 2003; John P. Sheehan & Walke, 2006).

Inhibition for human coagulation protease. Effects of compounds on coagulation factors were tested at the concentrations for inhibiting 50% and 90% iXase activity, in the presence or absence of AT-III using chromogenic substrate hydrolysis assays. Buffers used in the assays included 20 mM Tris/HCl buffer, pH 7.4, containing 100 mM NaCl, 2.5 mM $CaCl_2$, and 0.1% polyethylene glycol (PEG) 8000 for f.IIa and f.Xa assays (Henry, Monien *et al.* , 2007); 25 mM HEPES buffer, pH 7.4, containing 100 mM NaCl and 5 mM $CaCl_2$ for f.VIIa assays (Henry, Monien *et al.* , 2007; Lawson, Butenas *et al.* , 1993; Neuenschwander, Branam *et al.* , 1993); 100 mM HEPES buffer, pH 8.0, containing 100 mM NaCl and 10 mM $CaCl_2$ for f.IXa assays (Henry, Monien *et al.* , 2007; Tina, Richard *et al.* , 2003); 25 mM Tris/HCl buffer, pH 7.4, containing 100 mM NaCl, 0.1 mg/mL bovine serum albumin (BSA), 0.1% PEG 8000 for f.XIa assays (Likui, Mao-Fu *et al.* , 2009; Wuillemin, Eldering *et al.* , 1996) and 50 mM Tris/HCl buffer, pH 7.6, containing 150 mM NaCl, 0.1% BSA for f.XIIa assays (Tong, G Michael *et al.* , 2004).

The effects of compounds on coagulation factors in the presence of AT-III were assayed in 96-well plates in following conditions: 100 nM f.VIIa, 1000 nM AT-III and 1 mM Pefachrome f.VIIa for f.VIIa assay; 100 nM f.IXa, 1000 nM AT-III, 25% glycol and 1 mg/mL Pefchrome f.IXa for f.IXa assay; 5 nM f.XIa, 100 nM

AT-III and 1 mM S-2366 for f.XIa assay; 10 nM f.XIIa, 100 nM AT-III and 1 mM CS 31(02) for f.XIIa assay. The f.IIa and f.Xa assays were conducted according to instructions of heparin anti-f.IIa and -f.Xa test kits. In the assays without AT-III, the AT-III solutions were replaced with the same volume of buffer. After incubating the mixture for 1 min, chromogenic substrate was added, the OD_{405nm} was measured at 37°C (kinetic method, read per 15 s for 2 min).. The ΔOD_{405nm}/min in the presence of the test compounds was normalized to that of the control (compounds were replaced by buffer) to obtain the relative activity. Assays were carried out in duplicate.

Anticoagulant activities. APTT, PT, and TT were determined with a coagulometer (TECO MC-4000, Germany) using APTT, PT and TT reagents and human plasma as previously described (N. Gao, Wu *et al.* , 2012). In brief, 5 μL sample and 45 μL plasma were pipetted to cuvette; incubated at 37°C for 2 min, then 50 μL APTT reagent was added; after incubating for 3 min, 50 μL $CaCl_2$ reagent was added, and clotting time was recorded. The compound plasma concentrations-clotting times were fitted linearly by Origin 8.0 software (OriginLab, USA), and the $EC_{2.0\times}$ (concentration required for doubling clotting time) were calculated from the fitting curves. Determinations were performed in duplicate.

Rats deep venous thrombosis. The antithrombotic activities of dHG-5 oligosaccharides were tested at doses with equivalent anticoagulant to dHG-5: dHG-5 antithrombotic 50% effective dose (ED_{50}) × [oligo-saccharide $EC_{2.0\times}$ / dHG-5 $EC_{2.0\times}$]. Thrombus formation was induced by a combination of thromboplastin and stasis (Vogel, Meuleman *et al.* , 1989). Rats were anesthetized with intraperitoneal injection of 10% chloral hydrate (3 mL/kg). The abdomen was opened and the vena cava was exposed and dissected free from surrounding tissue. A loose suture was prepared under the inferior vena cava. 2% rabbit brain tissue thromboplastin suspension (1.5 mL/kg) was injected into femoral vein, after 10 s, stasis was established by tightening the suture and maintained for 20 min. Then ligated vessel (from the ligation site to 2 cm below) was opened longitudinally and the formed thrombus was taken, blotted on filter paper, dried at 60°C for 1 h and weighted (Mettler balance). Compounds or vehicle were administered subcutaneously 1 hour prior to the injection of thromboplastin. The rabbit brain tissue thromboplastin was prepared as previously described (Quick, 1936).

Mice tail-bleeding model. The doses of dHG-5 (73.01 mg/kg) and LMWH (37.49 mg/kg) were designed as ten times of their antithrombotic ED_{50}, respectively. Mice tail was cut 1 h after subcutaneous administration of compounds or vehicle. Mice were placed in prone position within an in-house device and a distal 5 mm segment of the tail was amputated. The tail was immediately immersed in a 50 mL pure water (37°C) for 1 h. After 1 h sample collection, the hemoglobin in the hemolyzed blood solution was measured using a UV-VIS spectrophotometer (UV-2450, Shimadzu) at 540 nm. Volume of blood loss was calculated from the OD_{540nm} - blood volume standard curve.

f.XII activation. The activation effects of compounds on f.XII were determined using chromogenic substrate hydrolysis assays (Wu, Wen*et al.* , 2015). 40 μL diluted plasma (1:3, plasma: Tris/HCl buffer (v/v)) and 30 μL compounds dissolved in Tris/HCl buffer (0.02 M, pH 7.4) were mixed and incubated at 37 for 1 min. Then 30 μL of 0.3 mM CS-31(02) was added. The activation effects of compounds on f.XII were positively correlated with the release of p-nitroaniline from each substrate, which was monitored every 15 s for 2 min at 405 nm on a microplate reader. The activation effects of compounds on f.XII expressed as ΔOD_{405nm}/min. OSCS was used as positive control. Determinations were performed in duplicate.

Platelet aggregation. Human blood was collected from elbow vein of healthy volunteers, anticoagulated with 3.2% sodium citrate, and centrifuged at 180 g for 10 min to obtain platelets rich plasma (PRP). The remaining components of the blood were centrifuged again at 1200 g for 15min to obtain platelets poor plasma (PPP). Rat blood were collected from abdominal aorta into tubes containing $EDTAK_2$, and centrifuged at 180 g for 10 min to obtain PRP. Then rat washed platelets (WP) was prepared (Cazenave, Ohlmann*et al.* , 2004; Mustard, Packham *et al.* , 1972). Briefly, platelets pellet were washed twice by Ca^{2+}-free Tyrode´s buffer (137 mM NaCl, 2.68 mM KCl, 0.2 mM $MgSO_4 \cdot 7H_2O$, 0.42 mM $NaH_2PO_4 \cdot 2H_2O$, 11.9 mM $NaHCO_3$, 5.05 mM Glucose, 0.2 mM EGTA, pH 6.53) for twice. After centrifugation (800 g, 10 min), platelet pellet was re-suspended in Tyrode´s buffer (137 mM NaCl, 2.68 mM KCl, 1.05 mM $MgSO_4 \cdot 7H_2O$,

0.42 mM $NaH_2PO_4 \cdot 2H_2O$, 11.9 mM $NaHCO_3$, 5.05 mM Glucose, 1.8 mM $CaCl_2$, pH 7.35). Light transmission aggregation was measured using an aggregometer (Model 700, Chrono-log, America). 250 μL PRP or WP suspension was stimulated with 2.5 μL compounds dissolved in saline for 10 min at 37°C and continuous stirring at 1000 rpm. The baselines were adjusted by PPP and Tyrode´s buffer for PRP and WP suspension, respectively. Determinations were performed in duplicate.

Preliminary pharmacodynamics study on mice. Compounds dissolved in saline were injected subcutaneously to mice. Venous blood samples (approximately 0.15 mL) were collected before and after compounds administration 0.5 h, 1 h, 2 h, 4 h and 8 h from mice orbital venous plexus and centrifuged immediately at 1800 g for 10 min. Then, the plasma APTT at different time points was analyzed ex vivo. In brief, 25 μL plasma sample was pipetted to cuvette, incubated at 37°C for 2 min, then 25 μL APTT reagent was added; after incubating 3 min, 25 μL $CaCl_2$ reagent was added and clotting time was recorded (MC-4000, MDC). All pharmacodynamics parameters were calculated with noncompartmental procedure using the Drug Analysis System 2.0 (DAS 2.0, BioVoice BioGuider, Shanghai, China).

Data and analysis

Data were expressed as mean ± standard deviation (SD). Numbers for every experiment (and group) are given in the figure legends and refer to the number of every individual animals used in the respective group. Data obtained from inappropriate measurements (e.g. mouse dying during blood sampling) were excluded. Data were analyzed by one-way analysis of variance followed by Tukey's test using the SPSS 17.0 (IBM, USA). Note that p-values less than 0.05 was considered to be statistically significant (i.e.,*p <0.05, **p< 0.01, or ***p <0.001). All data were analyzed without any transformation except the data from ED_{50} measurements, which were log-transformed.

Results

Native FG, dHG-5 and Oligosaccharides Contained in dHG-5.

Preparation of native FG, dHG-5 and its oligosaccharides. The native FG was extracted and purified from *H. fuscopunctata* with yield of ˜ 0.8 %. The[1]H NMR spectrum (Figure S1) showed native FG had a backbone consisted of repeated {4)-*D* -glucuronic acid (GlcA)-β(1,3)-N-acetyl-*D* -galactosamine (GalNAc)-β(1,} disaccharide units, and abundant α-*L* -fucose sulfate (FucS) branches linked to C3 of each GlcA residue in backbone. The main type of FucS branches in the native FG was*L* -fucose-3,4-disulfates (Fuc_{3S4S}), and the minor were*L* -fucose-2,4-disulfates (Fuc_{2S4S}) and*L* -fucose-4-sulfates (Fuc_{4S}). The depolymerized fraction, dHG-5, was prepared from native FG by β-eliminative depolymerization as previously described (Zhou, Gao *et al.* , 2020) and the high performance gel permeation chromatography (HPGPC) profiles of dHG-5 (Figure 1A and Figure S2) showed that dHG-5 was the mixture of oligosaccharides with different degree of polymerization (dp). The molar percentages of the main oligosaccharides (oHG-5, oHG-8, oHG-11, oHG-14, oHG-17, oHG-20, oHG-23, oHG-26, oHG-29 and those with dp > 29) in dHG-5 were estimated as 4.86, 17.39, 17.55, 15.93, 13.62, 11.22, 6.62, 4.74, 2.95 and 5.12%, respectively, according to the proportion of peak area in HPGPC. dHG-5 was further size-fractionated using GPC with Bio-Gel P6 and P10 columns (Bio-Rad) under monitor of HPGPC using a TSK $G2000SW_{XL}$ column, and nine homogeneous oligosaccharides were obtained (Figure 1A). The Mw of dHG-5 was calculated as 5236 Da, according to the calibration curve from the data of purified oligosaccharides using GPC software. Structures of dHG-5 and its purified oligosaccharide fractions were analysed by 1D/2D NMR and ESI-MS.

Structure of dHG-5 . According to the[1]H/^{13}C NMR spectra, the non-reducing ends of dHG-5 were $\Delta^{4,5}$-unsaturated glucuronic acids (ΔU) and the reducing terminals were the alditol of glucuronic acids (*D* -GlcA-ol, *L* -gulonic acid). The chemical structure of dHG-5 was further confirmed by analyzing its 1D/2D NMR spectra (Figure S3-S6). The position 4 and 6 of GalNAc were both sulfated ($GalNAc_{4S6S}$) according to signals of H4 and H6 that shifted downfield by approximately 0.5-0.7 ppm. Based on the cross-signals in ^{1}H-^{1}H ROESY and[1]H-^{13}C HMBC spectra and the anomeric proton-proton coupling constant ($^{3}J_{H-H}$) values of GlcA and $GalNAc_{4S6S,}$ GlcA and $GalNAc_{4S6S}$ residues were linked with alternating β1,3 and β1,4 glycosidic linkage. Likewise, the Fuc_{3S4S} branches were linked to the position 3 of GlcA residues through an

α1,3 glycosidic linkage.

Structure of oligosaccharides from dHG-5. The complete structural analysis of compound oHG-5, oHG-8, oHG-11, oHG-14 and oHG-17 was also conducted with full assignments of 1D/2D NMR spectra (Figure S7-S26). The ^{1}H and ^{13}C NMR spectra of oHG-8 and oHG-11 were shown as representatives in Figure 1B. For signals in ^{1}H-, ^{13}C- and 2D-NMR spectra of oHG-11, oHG-14, oHG-17 were similar except differences in integral area of corresponding signals (Figure S27), the 2D-NMR spectra of oHG-20, oHG-23, oHG-26 and oHG-29 weren't recorded.

The ^{1}H NMR spectrum of oHG-8 recorded in D_2O showed four well resolved single proton resonances in the 5.0-6.0 ppm region. The low-field signal at 5.68 ppm indicated a typical proton at position 4 of the unsaturated uronic acid residue (ΔU) resulting from β-eliminative cleavage (Vitor Hugo Pomin, 2013). The signals at 5.28 ppm and 5.20 ppm were anomeric proton signals of Fuc_{3S4S} residue (F) linked to GlcA (U) and Fuc_{3S4S} residue (dF) linked to ΔU, respectively. The signal at 5.02 ppm was anomeric proton signal of Fuc_{3S4S} residue (rF) linked to reducing termini (rU). The ^{1}H-^{1}H COSY spectrum provided eight complete spin connectivity information for each sugar residue (Figure S12). Signals of all four protons of ΔU residue were identified by starting from the signal at 5.68 ppm assigned to the H-4, the H-1, H-2 and H-3 signals were observed with chemical shifts of 4.846, 3.830, 4.426 ppm, respectively. All protons of other residues were also clearly identified in a similar manner by starting from their respective anomeric proton signals.

Total assignment of the carbon spectrum of oHG-8 (Figure S12) was achieved by analysis its ^{1}H-^{13}C HSQC (Figure S14). The C-1 chemical shifts of ΔU, dF, dA, F, U, A, rU and rF residues were at 105.81, 100.94, 102.39, 101.97, 106.44, 104.17, 65.21 and 104.17 ppm, respectively. The C4 and C5 of ΔU were at 109.44 ppm and 149.61 ppm, respectively, in accordance with the presence of double bond. The carbonyl of ΔU at 171.65 ppm shifted highfield by about 6 ppm. In addition, negative-ion electrospray ionization quadrupole time-of-flight mass spectrometry (ESI-Q-TOF MS) analysis revealed several multiply charged ions in the spectrum (Figure 1C). The four charged ions $[M-4Na]^{4-}$ with m/z at 591.9546 were observed as the most abundant ions. The molecular mass was identical to the calculated value of 2459.8184, confirming that the molecular formula of oHG-8 is $C_{52}H_{69}N_2Na_{13}O_{70}S_{10}$(octasaccharide). Therefore, oHG-8 was determined to be an octasaccharide with the sequence*L* -Fuc$_{3S4S}$-α(1,3)-*L* -Δ^{4,5}GlcA-α(1,3)-*D* -GalNAc$_{4S6S}$-β(1,4)-[*L* -Fuc$_{3S4S}$-α(1,]3)-*D* -GlcA-β(1,3)-*D* -GalNAc$_{4S6S}$-β(1,4)-[*L* -Fuc$_{3S4S}$-(α1,] 3)-*D* -GlcA-ol.

The structures of compound oHG-5, oHG-11, oHG-14 and oHG-17 were determined using the similar approach. They were identified as penta-, hendeca-, tetradeca- and heptadeca-saccharide by ^{1}H NMR spectra, respectively, based on the integral area ratio of the anomeric proton signals of Fuc_{3S4S}residues linked to different positions of their backbones (Figure S27). The main components of oHG-20, oHG-23, oHG-26, oHG-29 were eicosa-, tricosa-, hexacosa- and nonacosasaccharide, respectively, according to their HPGPC (Figure 1) and their ^{1}H NMR spectra (Figure S27). In short, oHG-5, oHG-8, oHG-11, oHG-14 and oHG-17 were highly regular oligosaccharides, and the non-reducing terminal of these compounds was ΔU produced by the β-eliminative cleavage. The internal sequence of these oligosaccharides was constituted by the repeating trisaccharide unit {4)-[*L* -Fuc-α(1,3)]-*D* -GlcA-β(1,3)-*D* -GalNAc-(β1,}, where Fuc side chains and GalNAc residues were primarily Fuc_{3S4S} and $GalNAc_{4S6S}$, respectively. It is worth noting that the reducing end was a hexuronic acid alditol residue instead of sulfated GalNAc, which were resulted from the peeling reaction (Shang, Gao *et al.* , 2018). The structural difference among these depolymerized oligo-saccharides was the number of the repeating trisaccharide unit {3)-*D* -GalNAc$_{4S6S}$-β(1,4)-[*L* -FucS-α(1,]3)-*D* -GlcA-β(1,}.

Potencies and Selectivity of Anti-iXase Activities of dHG-5 and its Containing Oliogosaccharides

Previous research showed that some depolymerized FG and oligosaccharides retained the potent activity for inhibiting iXase without the adverse effects of native FG (Zhao, Wu *et al.* , 2015). Herein, the anti-iXase activities and selectivity of dHG-5 and its containing oligosaccharides were investigated.

Anti-iXase activity. The activities of dHG-5 and its containing oligosaccharides in inhibiting iXase were evaluated based on their IC_{50} (Figure 2). dHG-5 had small IC_{50} of 14.0 nM of anti-iXase. For dHG-5

containing oligosaccharides, oHG-5 had the largest IC_{50} (> 3000 nM) of anti-iXase, the IC_{50} of anti-iXase of oligosaccharides with dp 8~14 decreased significantly (156 nM ~ 27.1 nM) with the extending of their chain length, while the IC_{50} of anti-iXase of oligosaccharides with dp 17~29 had small changes (17.2 nM ~ 2.93 nM), and the IC_{50} of anti-iXase of oHG-17 approximated to that of dHG-5 (Figure 2). The functional relationship between the Mw (x) and IC_{50} of anti-iXase of dHG-5 containing oligosaccharides (y) was fitted well with the power function (classical Freundlich model $y = a \times x^b$): $y = 1.66 \times 10^{13} \times x^{-3.25}$ ($R^2 = 0.997$) (Figure 2B). Further calculation showed that the anti-iXase potency of dHG-5 (100 U, used as standard) was close to the weighted average sum of that of its containing oligosaccharides (Table 1)

f.IXa-binding affinity. Previous studies found that FG oligosaccharides inhibited iXase activity by binding to f.IXa and disrupting the formation of iXase complex (John P. Sheehan & Walke, 2006; Xiao, Lian *et al.*, 2016; Xiao, Zhao *et al.*, 2019). Herein, the f.IXa-binding affinity of dHG-5 and its containing oligosaccharides were assessed by the competition study using bio-layer interferometry (BLI). The kinetics parameters of f.IXa-oHG-11 binding were determined (Figure 3A). The interaction of f.IXa and oHG-11 fitted well with the 2:1 heterogeneous ligand binding model, with high affinity (Table S1).

The abilities of dHG-5 containing oligosaccharides competitively binding to f.IXa (100 nM) were evaluated by determining their IC_{50} values for inhibiting f.IXa to bind to immobilized oHG-11 (Figure 3B). dHG-5 had small IC_{50} of 0.430 μM of f.IXa-binding. For dHG-5 containing oligosaccharides, oHG-5 had the largest IC_{50} (~ 12.9 μM) of f.IXa-binding, the IC_{50} of f.IXa-binding of oligosaccharides with dp 8~14 decreased significantly (3.29 μM ~ 1.20 μM) with the extending of their chain length, while the IC_{50} of f.IXa-binding of oligosaccharides with dp 17~29 were similar (0.416 μM ~ 0.284 μM), and the IC_{50} of f.IXa-binding of oHG-17 approximated to that of dHG-5. The functional relationship between the Mw (x) and IC_{50} of f.IXa-binding of dHG-5 containing oligosaccharides (y) was fitted well with the power function: $y = 3.36 \times 10^{10} \times x^{-2.06}$ ($R^2 = 0.970$) (Figure 3C). Further calculation showed that the f.IXa-binding potency of dHG-5 (100 U, used as standard) was close to the weighted average sum of that of its containing oligosaccharides (Table 1)

Effects of dHG-5 and its oligosaccharides on other coagulation factor targets. To investigate the anti-iXase selectivity of dHG-5 and its containing oligosaccharides, their effects on f.IIa, f.VIIa, TF-f.VIIa, f.Xa, f.XIa and f.XIIa in the presence or absence of AT-III were assayed. Concentrations of dHG-5 and its containing oligosaccharides that resulted in 50% or 90% iXase inhibition were used in assays.

At the concentrations of inhibiting 50% iXase, dHG-5 and its containing oligosaccharides showed no obvious effects on the activities of f.IIa, f.VIIa, f.Xa, f.XIa and f.XIIa in the presence or absence of AT-III (Figure 4A), except that oHG-5 (had weakest anti-iXase activity) obviously inhibited f.VIIa in the absence of AT-III (Figure 4B). At much higher concentrations of inhibiting 90% iXase, in the presence of AT-III, dHG-5 and its containing oligosaccharides showed little or very weak effects on f.IIa, f.XIa and f.XIIa (Figure 4C-D). Although oHG-5, -8, -11, and -14 showed slight to moderate inhibition (14.2% ~ 41.8%) of f.Xa (Figure 4C-D), dHG-5 and oligosaccharides with dp > 14 had no obvious effects on f.Xa. While dHG-5 and its containing oligosaccharides enhanced the activity of f.IXa in hydrolyzing its chromogenic substrate in the absence of f.VIIIa (Figure 4E-F).

Anticoagulant and Thrombotic Activities of dHG-5 and its Containing Oliogosaccharides

Anticoagulant activity. The anticoagulant activities of dHG-5 and its oligosaccharides were evaluated as their effects on APTT, PT and TT of human coagulation control plasma.

dHG-5 had small $EC_{2.0\times}$. For dHG-5 containing oligosaccharides, oHG-5 had the largest $EC_{2.0\times}$, the $EC_{2.0\times}$ of oligosaccharides with dp 8~14 decreased significantly with the extending of their chain length, while the $EC_{2.0\times}$ of oligosaccharides with dp 17~29 had no large change, and the $EC_{2.0\times}$ of oHG-17 approximated to that of dHG-5 (Figure 5B). dHG-5 and its oligosaccharides had no obvious effects on plasma PT and TT (Table S2). The relationship between Mw (x) and $EC_{2.0\times}$ of dHG-5 containing oligosaccharides (y) was fitted well with the power function: $y = 1.33 \times 10^{11} \times x^{-2.92}$ ($R^2 = 0.999$) (Figure 5B). Further calculation showed that the anticoagulant potency of dHG-5 (100 U, used as standard) was close to the weighted average sum of that of its containing oligosaccharides (Table 1)

14

Nagase H. reported that dFG had the anti-f.IIa activity in the presence of heparin cofactor II (HCII) besides anti-iXase activity (Nagase, Enjyoji *et al.*, 1995), our group also confirmed this (Wu, Wen*et al.*, 2015). To evaluate the effects of AT-III and HCII on the anticoagulant activities of dHG-5 and its containing oligosaccharides, AT-III- or HCII-deficient plasmas were used for assays. Compared with AT-III-deficient plasma, oHG-5, oHG-17, oHG-29 and dHG-5 didn't increase the APTT of AT-III-complementary plasma. And the APTT-prolonging activities of oHG-8, oHG-17, oHG-29 and dHG-5 in HCII-deficient plasma approached to those in coagulation control plasma (Figure 5C-D).

Antithrombotic activity. The antithrombotic activities of dHG-5 and its containing oligosaccharides were evaluated using the rat deep venous thrombosis model.

The results showed that after subcutaneously injecting, dHG-5 dose-dependently inhibit the venous thrombosis induced by stasis and thromboplastin, with the ED_{50} of 2.38 mg/kg (Figure 6A). To further compare the antithrombotic activities of dHG-5 containing oligosaccharides, their antithrombotic activities were evaluated at the equivalent anticoagulant doses. The result showed that, at the equivalent anticoagulant doses, the thrombosis inhibition effects of these oligosaccharides were not significantly different among treated groups, and closed to 50% except oHG-5 and -29 (Figure 6B). Further calculation showed that the antithrombotic potency of dHG-5 (100 U, used as standard) was close to the weighted average sum of that of its containing oligosaccharides (Table 1).

Relative Pharmacological Properties

Bleeding risk. Bleeding risk has been the main problem in the application of anticoagulants. Herein, the effect of dHG-5 on bleeding was assessed by the mice tail bleeding model. At 10 times anti-thrombosis ED_{50} doses, dHG-5 did not significantly increase the blood loss compared with control group (Figure 6C).

f.XII and platelets activation. Native FG had the undesired activation on f.XII and platelets (J. Z. Li, Bao *et al.*, 1985), which may result in proinflammation or thrombocytopenia, respectively. Therefore, the activation of dHG-5 and its containing oligosaccharides (oHG-8, oHG-14 and oHG-29) on f.XII and platelets were analyzed.

The results showed that dHG-5 and its containing oligosaccharides had no obvious activation on f.XII at the concentrations up to 10 μM (Figure 7A). At the peak concentrations of the bleeding tolerance doses (Figure S28) dHG-5, oHG-8, oHG-14 and oHG-29 showed no obvious activation on platelets, either in human PRP or rat WP, whereas FG could induce platelet aggregation (Figure 7B-C).

Preliminary pharmacodynamics. After administrating subcutaneously to mice, the effect of dHG-5 on plasma APTT was determined (Figure 8). And the pharmacodynamic parameters were calculated according to noncompartmental procedure model (Table S3). The the maximum APTT-prolonging effect (E_{max}) and AUC_{0-8h} (the area under the time-effect curve within 8 h) increased in a dose-proportional manner, while the half-life ($T_{1/2}$) did not obviously change (Figure 8 and Table S3).

DISSCUSSION

Assay for potencies and selectivity of anti-iXase activities of dHG-5 and its containing oliogosaccharides showed that dHG-5 showed potent anti-iXase activity and f.IXa-binding activity without obvious effects on the other coagulation factor targets, which indicated its high selectivity in anti-iXase. Silmilar to our previous report (Xiao, Zhao*et al.*, 2019), it was notable that dHG-5 and its containing oligosaccharides seemed to enhance the activity of f.IXa in hydrolyzing its chromogenic substrate in the absence of f.VIIIa. This phenomena might be attributed to the conformational change of f.IXa induced by oligosaccharides. In fact, under physiological conditions, f.IXa combines its cofactor f.VIIIa, phospholipid and $Ca^{2+}$to form iXase complex, which enhances the catalytic efficiency of f.IXa with million times (Duffy & Lollar, 1992; Mertens, Wijngaarden *et al.*, 1985). Thus, the direct effects of dHG-5 and its containing oligosaccharides on f.IXa activity was very faint when compared with their inhibition on f.IXa-f.VIIIa complex assembling, i.e., anti-iXase activities.

15

Assay for anticoagulant activities of dHG-5 and its containing oliogosaccharides showed that the anticoagulant mechanisms of dHG-5 and its containing oligosaccharides were AT-III- and HCII-independent, which were different from that of LMWH and DS. At the equivalent anticoagulant potency's doses, these oligosaccharides showed approximate thrombosis inhibition, suggesting their antithrombotic activities were closely related to their anticoagulant activities. The results of pharmacodynamics of dHG-5 showed the linear kinetics, suggesting the predictable pharmacodynamic characteristics.

Through a series of studies, the pharmacological properties of dHG-5 and its containing oligosaccharides in anti-iXase, f.IXa-binding, anticoagulant and antithrombotic activities were clearly illuminated. dHG-5 showed strong anti-iXase activity, f.IXa-binding, anticoagulant and antithrombotic activities without activating f.XII and platelets. Within bounds, the anti-iXase, f.IXa-binding and anticoagulant activities of these oligosaccharides increased with the increase of dp, oHG-17 to -29 closed to the full activity. It indicated that the increase of dp obviously increased these activities of these oligosaccharides to a certain extent, even octasaccharide was the minimum structural unit required for the potent anti-iXase activity (Yin, Zhou *et al.* , 2018). Moreover, the activity potencies of dHG-5 in anti-iXase, f.IXa-binding, anticoagulation and anti-thrombosis were close to the weighted average sum of that of its containing oligosaccharides (Table 1). It indicated that there was no synergy or antagonism among these oligosaccharides, the similarity of dHG-5 and oHG-17 in Mw and activities also confirmed this. Thus pharmacological activities of dHG-5 could be explained well by the contribution of its containing oligosaccharides both qualitatively and quantitatively. Besides, the relationships between Mw and f.IXa-binding, anti-iXase, anticoagulant and antithrombotic potencies of dHG-5 containing oligosaccharides all fitted well with similar power function, which suggested the high dependency among these activities.

dHG-5 is an active ingredient consisting of a series of oligosaccharides homologs. As a multi-component drug, it is a great challenge to clarify the correlation of pharmacological activity between drug and its containing components. For instance, heparins and LMWHs have been widely used in clinic, but due to complexity of the composition, the types and composition ratios of their containing oligosaccharides could not be clarified (Loganathan, Wang *et al.* , 1990). Consequently, the correlation of pharmacological activity between these preparations and their containing oligosaccharides has not been reported. Our study showed that the chemical composition of dHG-5 was relatively clear. And the nine purified oligosaccharide components (oHG-5, -8, -11, -14, -17, -20, -23, -26 and -29) accounted for about 95% of dHG-5. And spectral analysis (1D/2D NMR and MS) confirmed that these oligosaccharide components had the regular structures and shared the common formula. Compared with heparins that have complex composition and diverse substituents, the chemical composition of dHG-5 was clear, which enabled the further study in structure-activity relationship and the correlation of pharmacological activities between dHG-5 and its containing oligosaccharides.

According to pharmacoeconomics, dHG-5 is suitable for developing a noval anticoagulant. Though the activities of oHG-17 are similar to that of dHG-5, the preparation process of pure oligosaccharide is complex, and not feasible for large-scale industrial production. While it is more feasible for the scale preparation of dHG-5. What's more, the composition and proportion of dHG-5 containing oligosaccharides could be controlled well in the preparation process. Therefore, dHG-5 may be more suitable as an iXase inhibitor to be a novel anticoagulant applied in clinic.

dHG-5 may be more effective than other intrinsic coagulation inhibitors in antithrombosis. The effects of common coagulation pathway inhibitors and intrinsic coagulation pathway inhibitors on hemostatic function are different (Colman, 2006; Qiufang, Tucker *et al.* , 2010; Wheeler & Gailani, 2016; Woodruff, Xu *et al.* , 2013). Compared with available clinical drugs, selective intrinsic coagulation pathway inhibitors may have the characteristics of antithrombosis with low bleeding tendency (Lin, Zhao *et al.* , 2020). According to the cell-based coagulation model, f.XIIa and f.XIa inhibitors may have limited effects on coagulation amplification and propagation during thrombosis, while iXase inhibitors should be able to exhibitie more effective antithrombotic activity (Hoffman, 2003; Lin, Zhao *et al.* , 2020).

In conclusion, our data demonstrate the anti-iXase, f.IXa-binding, anticoagulant and antithrombotic activities of dHG-5 are contributed by that of its containing oligosaccharides in terms of a weighted average sum.

These activities of dHG-5 containing oligosaccharides were positively correlated with their chain length to a certain degree, among which, the molecular weight of oHG-17 may be necessary to achieve full activity. Not only dHG-5 has the characteristic of antithrombosis with low bleeding tendency, but also clear chemical composition. This paper makes an important supplement to the preclinical study of dHG-5, which makes a good preparation for the entry into the clinical study.

Reference

Ahmad, S. S.,Rawala-Sheikh, R.Walsh, P. N. (1992). Components and Assembly of the Factor X Activating Complex. *Seminars in Thrombosis and Hemostasis, 18* (03), 311-323.

Becattini, C.,Cohen, A. T.,Agnelli, G.,Howard, L.,Castejon, B.,Trujillo-Santos, J.*, et al.* (2016). Risk stratification of patients with acute symptomatic pulmonary embolism based on presence or absence of lower extremity DVT systematic review and meta-analysis.*Chest, 149* (1), 192-200.

Benjamin, E. J.,Virani, S. S.,Callaway, C. W.,Chamberlain, A. M.,Chang, A. R.,Cheng, S.*, et al.* (2018). Heart Disease and Stroke Statistics-2018 Update: A Report from the American Heart Association.*Circulation, 137* (12), e67-e492.

Cai, Y.,Yang, W. J.,Li, X. M.,Zhou, L. T.,Wang, Z. J.,Lin, L. S.*, et al.* (2019). Precise Structures and Anti-intrinsic Tenase Complex Activity of Three Fucosylated Glycosaminoglycans and Their Fragments.*Carbohydrate Polymers, 224* , 115146.

Campello, E.,Henderson, M. W.,Noubouossie, D. F.,Simioni, P.Key, N. S. (2018). Contact system activation and cancer: new insights in the pathophysiology of cancer-associated thrombosis. *Thrombosis and Haemostasis, 118* (2), 251-265.

Carpenter, S. L.,Richardson, T.Hall, M. (2018). Increasing Rate of Pulmonary Embolism Diagnosed in Hospitalized Children in the United States from 2001 to 2014. *Blood Advances, 2* (12), 1403-1408.

Cazenave, J. P.,Ohlmann, P.,Cassel, D.,Eckly, A.,Hechler, B.Gachet, C. (2004). Preparation of Washed Platelet Suspensions from Human and Rodent Blood. *Methods Mol. Biol. (Clifton, N.J.), 272* , 13-28.

Colman, R. W. (2006). Are hemostasis and thrombosis two sides of the same coin? *Journal of Experimental Medicine, 203* (3), 493-495.

Duffy, E. J.Lollar, P. (1992). Intrinsic pathway activation of factor X and its activation peptide-deficient derivative, factor Xdes-143-191.*Journal of Biological Chemistry, 267* (11), 7821-7827.

Figueralosada, M.Lograsso, P. V. (2012). Enzyme Kinetics and Interaction Studies for Human JNK1β1 and Substrates Activating Transcription Factor 2 (ATF2) and c-Jun N-terminal Kinase (c-Jun). *Journal of Biological Chemistry, 287* (16), 13291-13302.

Fonseca, R. J. C.,Santos, G. R. C.Mour, O. P. A. S. (2009). Effects of Polysaccharides Enriched in 2,4-Disulfated Fucose Units on Coagulation, Thrombosis and Bleeding. Practical and Conceptual Implications.*Thrombosis and Haemostasis, 102* (05), 829-836.

Gao, N.,Lu, F.,Xiao, C.,Yang, L.,Chen, J.,Zhou, K.*, et al.*(2015). Beta-eliminative Depolymerization of the Fucosylated Chondroitin Sulfate and Anticoagulant Activities of Resulting Fragments.*Carbohydrate Polymers, 127* , 427-437.

Gao, N.,Wu, M.,Liu, S.,Lian, W.,Li, Z.Zhao, J. (2012). Preparation and Characterization of O-acylated Fucosylated Chondroitin Sulfate from Sea Cucumber. *Marine Drugs, 10* (8), 1647-1661.

Henry, B. L.,Monien, B. H.,Bock, P. E.Desai, U. R. (2007). A Novel Allosteric Pathway of Thrombin Inhibition: Exosite II Mediated Potent Inhibition of Thrombin by Chemo-enzymatic, Sulfated Dehydropolymers of 4-hydroxycinnamic Acids. *Journal of Biological Chemistry, 282* (44), 31891-31899.

Hoffman, M. (2003). A cell-based model of coagulation and the role of factor VIIa. *Blood Reviews, 17* (1), S1-S5.

Jiménez, D.,Bikdeli, B.,Quezada, A.,Muriel, A.Monreal, M. (2019). Hospital Volume and Outcomes for Acute Pulmonary Embolism: Multinational Population Based Cohort Study. *BMJ Clinical Research, 366* , l4416.

Kitazato, K.,Kitazato, K. T.,Nagase, H.Minamiguchi, K. (1996). DHG, a new depolymerized holothurian glycosaminoglycan, exerts an antithrombotic effect with less bleeding than unfractionated or low molecular weight heparin, in rats. *Thrombosis Research, 84* (2), 111-120.

Lawson, J. H.,Butenas, S.,Ribarik, N.Mann, K. G. (1993). Complex-dependent inhibition of factor VIIa by antithrombin III and heparin. *Journal of Biological Chemistry, 268* (2), 767-770.

Li, B.,Suwan, J.,Martin, J. G.,Zhang, F.,Zhang, Z.,Hoppensteadt, D.*, et al.* (2009). Oversulfated chondroitin sulfate interaction with heparin-binding proteins: new insights into adverse reactions from contaminated heparins. *Biochemical Pharmacology, 78* (3), 292-300.

Li, J. Z.,Bao, C. X.,Chen, G. Z.,Zhang, G. Z.,Fan, H. Z.Chen, J. D. (1985). Antithrombin activity and platelet aggregation by acid mucopolysaccharides isolated from Stichopus Japonicus Selenka.*Acta Pharmacologica Sinica, 6* (2), 107-110.

Li, X. M.,Luo, L.,Cai, Y.,Yang, W. J.,Lin, L. S.,Li, Z.*, et al.*(2017). Structural Elucidation and Biological Activity of A Highly Regular Fucosylated Glycosaminoglycan from the Edible Sea Cucumber*Stichopus Herrmanni* . *Journal of Agricultural and Food Chemistry, 65* (42), 9315-9323.

Likui, Y.,Mao-Fu, S.,David, G.Rezaie, A. R. (2009). Characterization of a heparin-binding site on the catalytic domain of factor XIa: mechanism of heparin acceleration of factor XIa inhibition by the serpins antithrombin and C1-inhibitor. *Biochemistry, 48* (7), 1517-1524.

Lin, L.,Zhao, L.,Gao, N.,Yin, R.,Li, S.,Sun, H.*, et al.* (2020). From multi-target anticoagulants to DOACs, and intrinsic coagulation factor inhibitors. *Blood Reviews, 39* , 100615.

Loganathan, D.,Wang, H. M.,Mallis, L. M.Linhardt, R. J. (1990). Structural Variation in the Antithrombin III Binding Site Region and its Occurrence in Heparin from Different Sources. *Biochemistry, 29* (18), 4362-4368.

Long, A. T.,Kenne, E.,Jung, R.,Fuchs, T. A.Renne, T. (2016). Contact system revisited: an interface between inflammation, coagulation, and innateimmunity. *Journal of Thrombosis and Haemostasis, 14* (3), 427-437.

Mertens, K.,Wijngaarden, A. V.Bertina, R. M. (1985). The role of factor VIII in the activation of human blood coagulation factor X by activated factor IX. *Thrombosis and Haemostasis, 54* (3), 654-660.

Mustard, J. F.,Packham, M. A.,Perry, D. W.Ardlie, N. G. (1972). Preparation of Suspensions of Washed Platelets from Humans.*British Journal of Haematology, 22* (2), 193-204.

Nagase, H.,Enjyoji, K.,Minamiguchi, K.,Kitazato, K. T.,Kitazato, K.,Saito, H.*, et al.* (1995). Depolymerized holothurian glycosaminoglycan with novel anticoagulant actions: antithrombin III- and heparin cofactor II-independent inhibition of factor X activation by factor IXa-factor VIIIa complex and heparin cofactor II-dependent inhibition of thrombi. *Blood, 85* (6), 1527-1534.

Neuenschwander, P. F.,Branam, D. E.Morrissey, J. H. (1993). Importance of substrate composition, pH and other variables on tissue factor enhancement of factor VIIa activity. *Thrombosis and Haemostasis, 70* (06), 0970-0977.

Pinto, D. J. P.,Orwat, M. J.,Smith II, L. M.,Quan, M. L.,Lam, P. Y. S.,Rossi, K. A.*, et al.* (2017). Discovery of a Parenteral Small Molecule Coagulation Factor XIa Inhibitor Clinical Candidate (BMS-962212). *Journal of Medicinal Chemistry, 60* , 9703-9723.

Pomin, V. H. (2013). NMR Chemical Shifts in Structural Biology of Glycosaminoglycans. *Analytical Chemistry, 86* (1), 65-94.

Pomin, V. H. (2014). Holothurian fucosylated chondroitin sulfate. *Marine Drugs, 12* (1), 232-254.

Qiufang, C.,Tucker, E. I.,Pine, M. S.,India, S.,Anton, M.,Mao-Fu, S.*, et al.* (2010). A role for factor XIIa-mediated factor XI activation in thrombus formation in vivo. *Blood, 116* (19), 3981-3989.

Quan, M. L.,Pinto, D. J. P.,Smallheer, J. M.,Ewing, W. R.,Rossi, K. A.,Luettgen, J. M.*, et al.* (2018). Factor XIa Inhibitors as New Anticoagulants. *Journal of Medicinal Chemistry, 61* , 7425-7447.

Quick, A. J. (1936). On Various Properties of Thromboplastin (Aqueous Tissue Extracts). *American Journal of Physiology, 114* (2), 282-296.

Robert, S.,Bertolla, C.,Masereel, B.,Dogné, J. M.Pochet, L. (2008). Novel 3-Carboxamide-coumarins as Potent and Selective FXIIa Inhibitors. *Journal of Medicinal Chemistry, 51* (11), 3077-3080.

Shang, F.,Gao, N.,Yin, R.,Lin, L.,Xiao, C.,Zhou, L.*, et al.*(2018). Precise Structures of Fucosylated Glycosaminoglycan and its Oligosaccharides as Novel Intrinsic Factor Xase Inhibitors. *European Journal of Medicinal Chemistry, 148* , 423-435.

Sheehan, J. P.,Kobbervig, C. E.Kirkpatrick, H. M. (2003). Heparin Inhibits the Intrinsic Tenase Complex by Interacting with an Exosite on Factor IXa. *Biochemistry, 42* (38), 11316-11325.

Sheehan, J. P.Walke, E. N. (2006). Depolymerized Holothurian Glycosaminoglycan and Heparin Inhibit the Intrinsic Tenase Complex by a Common Antithrombin-independent Mechanism. *Blood, 107* (10), 3876-3882.

Smith. (2010). The cell-based model of coagulation. *Journal of Veterinary Emergency & Critical Care, 19* (1), 3-10.

Timmis, A.,Townsend, N.,Gale, C.,Grobbee, R.,Maniadakis, N.,Flather, M.*, et al.* (2017). European society of cardiology: cardiovascular disease statistics 2017. *European Heart Journal, 39* (7), 508-579.

Tina, B.,Richard, S.,Yung-Jen, C.,Bock, P. E.,Ingemar, B. R.Olson, S. T. (2003). Heparin and calcium ions dramatically enhance antithrombin reactivity with factor IXa by generating new interaction exosites. *Biochemistry, 42* (27), 8143-8152.

Tong, S.,G Michael, I.,Jian, C., Qi,Cockerill, K. A.,Linnik, M. D.,Pamela, K.*, et al.* (2004). Beta 2-Glycoprotein I binds factor XI and inhibits its activation by thrombin and factor XIIa: loss of inhibition by clipped beta 2-glycoprotein I. *Proceedings of the National Academy of Sciences of the United States of America, 101* (11), 3939-3944.

Vogel, G. M. T.,Meuleman, D. G.,Bourgondi?n, F. G. M.Hobbelen, P. M. J. (1989). Comparison of Two Experimental Thrombosis Models in Rats Effects of Four Glycosaminoglycans. *Thrombosis Research, 54* (5), 399-410.

Wang, S.,Beck, R.,Burd, A.,Blench, T.,Marlin, F.,Ayele, T.*, et al.* (2010). Structure Based Drug Design: Development of Potent and Selective Factor IXa (FIXa) Inhibitors. *Journal of Medicinal Chemistry, 53* (4), 1473-1482.

Wheeler, A. P.Gailani, D. (2016). The intrinsic pathway of coagulation as a target for antithrombotic therapy. *Hematology/Oncology Clinics of North America, 30* (5), 1099-1114.

Woodruff, R. S.,Xu, Y.,Layzer, J.,Wu, W.,Ogletree, M. L.Sullenger, B. A. (2013). Inhibiting the intrinsic pathway of coagulation with a factor XII-targeting RNA aptamer. *Journal of Thrombosis and Haemostasis, 11* (7), 1364-1373.

Wu, M.,Wen, D.,Gao, N.,Xiao, C.,Yang, L.,Xu, L.*, et al.* (2015). Anticoagulant and antithrombotic evaluation of native fucosylated chondroitin sulfates and their derivatives as selective inhibitors of intrinsic factor Xase. *European Journal of Medicinal Chemistry, 92* , 257-269.

Wuillemin, W. A.,Eldering, E.,Citarella, F.,Ruig, C. P. D.,Cate, H. T.Hack, C. E. (1996). Modulation of Contact System Proteases by Glycosaminoglycans - Selective Enhancement of the Inhibition of Factor XIa. *Journal of Biological Chemistry, 271* (22), 12913-12918.

Xiao, C.,Lian, W.,Zhou, L.,Gao, N.,Xu, L.,Chen, J., *et al.*(2016). Interactions between depolymerized fucosylated glycosaminoglycan and coagulation proteases or inhibitors. *Thrombosis Research, 146* , 59-68.

Xiao, C.,Zhao, L. Y.,Gao, N.,Wu, M. Y.Zhao, J. H. (2019). Nonasaccharide Inhibits Intrinsic Factor Xase Complex by Binding to Factor IXa and Disrupting Factor IXa-Factor VIIIa Interactions. *Thrombosis and Haemostasis, 119* (5), 705-715. doi:10.1055/s-0039-1681047

Yin, R.,Zhou, L.,Gao, N.,Z., L.,Zhao, L.,Shang, F., *et al.*(2018). Oligosaccharides from depolymerized fucosylated glycosaminoglycan: structures and minimum size for intrinsic factor Xase complex inhibition. *Journal of Biological Chemistry, 293* , 14089-14099.

Zhao, L.,Wu, M.,Xiao, C.,Yang, L.,Lian, W.,Zhou, L., *et al.*(2015). Discovery of an Intrinsic Tenase Complex Inhibitor: Pure Nonasaccharide from Fucosylated Glycosaminoglycan. *Proceedings of the National Academy of Sciences of the United States of America, 112* (27), 8284-8289.

Zhou, L.,Gao, N.,Sun, H.,Xiao, C.,Yang, L.,Lin, L., *et al.*(2020). Effects of Native Fucosylated Glycosaminoglycan, its Depolymerized Derivatives on Intrinsic Factor Xase, Coagulation, Thrombosis and Hemorrhagic Risk. *Thrombosis and Haemostasis* . doi:10.1055/s-0040-1708480

Hosted file

`Figures.docx` available at https://authorea.com/users/310487/articles/441353-the-components-and-activities-analysis-of-noval-anticoagulant-candidate-dhg-5

Hosted file

`Table.docx` available at https://authorea.com/users/310487/articles/441353-the-components-and-activities-analysis-of-noval-anticoagulant-candidate-dhg-5