Original article

Anticoagulant and antithrombotic evaluation of native fucosylated chondroitin sulfates and their derivatives as selective inhibitors of intrinsic factor Xase

Mingyi Wu a,2, Dandan Wen a,b,1,2, Na Gao a, Chuang Xiao a,c, Lian Yang a, Li Xu a, Wu Lian a,c, Wenlie Peng c, Jianmin Jiang b, Jinhua Zhao a, *

a State Key Laboratory of Phytochemistry and Plant Resources in West China, Kunming Institute of Botany, Chinese Academy of Sciences, Kunming 650201, China
b School of Pharmaceutical Sciences, Sun Yat-sen University, Guangzhou 510006, China
c College of Life Sciences, Sun Yat-sen University, Guangzhou 510006, China

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ABSTRACT

Fucosylated chondroitin sulfate (FCS), a structurally unusual glycosaminoglycan, has distinct anticoagulant properties, and is an especially strong inhibitor of the intrinsic factor Xase (anti-Xase). To obtain a highly selective inhibitor of human Xase, we purified six native FCSs with various sulfation patterns, prepared a series of FCS derivatives, and then elucidated the relationship between the structures and the anticoagulant activities of FCSs. FCSs 1–3 containing higher Fuc2S4S exhibit stronger AT-dependent anti-IIa activities, whereas 4–6 containing more Fuc2S4S produce potent HCII-dependent anti-IIa activities. Saccharides containing a minimum of 6–8 trisaccharide units, free carboxyl groups, and full fucosylation of GlcA may be required for potent anti-Xase activity, and approximately six trisaccharide units and partial fucosylation of GlcA may contribute to potent HCII-dependent activity. Decreasing of the molecular weights markedly reduces their AT-dependent anti-IIa activities, and even eliminates human platelet and factor XII activation. Furthermore, in vitro and in vivo studies suggested that fractions of 6–12 kDa may be very promising compounds as putative selective intrinsic Xase inhibitors with antithrombotic action, but without the consequences of major bleeding and factor XII activation.

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1. Introduction

Thromboembolic diseases continue to be the leading cause of death throughout the world. Unfractionated heparin (UFH) and low molecular weight heparins (LMWHs) have been the clinical cornerstones of antithrombotic treatment and prophylaxis for the past 70 years, but the risk of hemorrhagic complications is still a major concern with their use [1].

Heparin and LMWHs are heterogeneous mixtures of poly- or oligo-saccharide chains with multiple potential anticoagulant mechanisms. Antithrombin (AT)-dependent mechanisms accelerate the inhibition of coagulation proteases by conformational activation of serpin, which requires a specific pentasaccharide sequence in heparin [2,3], or by binding serpin and protease to the same chain (template mechanism), which needs a longer sequence (>18 sugar units, including the pentasaccharide) [4,5].

Prolonged bleeding caused by heparin and LMWHs in animals has been proposed to be correlated to antithrombin activity, and AT-independent activities may be related to a lower bleeding risk in vivo [6]. In recent decades, a series of studies has demonstrated that inhibitors of the activated coagulant factors in the intrinsic pathway, such as factors IXa, Xla, and XIIla, should effectively prevent thrombus formation, with negligible bleeding risks [7,8].
In particular, factor IXa is a component of intrinsic factor Xase (factor IXa–VIIa complex). In vitro and in vivo models of the coagulation cascade indicate that factor X activation by factor Xase is the rate-limiting step in thrombin generation [9,10]. Given the critical role of intrinsic factor Xase, it would seem an ideal target for antithrombotic agents [11].

Fucosylated chondroitin sulfate (FCS), an unusual glycosaminoglycan derived from sea cucumber, possesses chondroitin sulfate-like backbones, and is markedly different to typical mammalian glycosaminoglycan because of its unique sulfated fucose side chains (Fig. 1) [12]. Several FCSs from sea cucumbers, such as Stichopus japonicus (also named Apostichopus japonicus), Ludwigothurea grisea, and Thelenata ananas, have been demonstrated to have potent anticoagulant activities [13–15]. Although their anticoagulant activities may be related to some mechanisms, such as heparin cofactor II (HCII) dependent inhibition of thrombin (factor IIa) [16–18], inhibition of factor VIII formation by factor IIa, and AT-dependent inhibition of thrombin [16,19], the primary mechanism may be inhibition of factor Xa generation by the intrinsic tenase complex [16,18,20]. Additionally, a depolymerized FCS has been demonstrated to inhibit plasma thrombin generation primarily by reducing prothrombin activation, rather than by accelerating the inhibition of thrombin by HPCI [21]. Therefore, its antithrombotic effect seems to be achieved mainly by the inhibition of factor Xase [22]. Although native FCS causes prolonged bleeding times in rabbits and mice [23–25], a depolymerized FCS has been found to exert an antithrombotic effect with less bleeding than UFH and LMWH in rats and dogs [26,27]. Therefore, it is very encouraging to discover Xase inhibitors based on FCSs, which are novel anticoagulants, selectively, or mainly, inhibiting the intrinsic coagulation pathway.

Another important consideration of FCSs as new anticoagulants is that previous studies have revealed that a native FCS, derived from *Stichopus japonicus*, induces clumping of human, rabbit, rat, and mouse platelets, and also causes a dramatic decrease in the platelet count and spontaneous bleeding after intravenous injection into rabbits and mice [25,28]; a native FCS from *L. grisea* can activate factor XII strongly and induce hypotension when injected intravenously into rats [24,29]. Clearly, factor XII and platelet activation would be a great hindrance to any potential use of native FCSs.

Some preliminary studies have been carried out comparing the pharmacological characteristics of FCSs with the different structures or their derivatives; for example, the anticoagulant activities of partially deucosylated, carboxyl-reduced FCS derivatives from *L. grisea* and several FCS subfractions have been inter-compared [17,23,30,31]. However, these reports did not indicate the effects of specific structures of FCSs, such as the sulfation pattern, the molecular chain length, and functional groups, on the potency and selectivity of their anticoagulant activities, especially on factor Xase inhibition and factor XII and platelet activation.

In the present study, native FCSs (compounds 1–6) from six sea cucumber species with various regular sulfation patterns of branches and backbones, their depolymerized fragments (7–12) with similar molecular weights (Mw), a series of depolymerized FCSs with gradually decreasing Mw (13–21), and several FCS derivatives (22–27) with different chemical modifications on functional groups (Scheme 1) were prepared. Then, structure–function relationships were elucidated in detail to find novel anticoagulants, based on FCS derivatives, derivatives with greater specificity for the target proteases of intrinsic Xase, but with lower factor XII and platelet activation activity. Furthermore, the antithrombotic and bleeding effects of the FCS derivatives, which were effective intrinsic Xase inhibitors, were assessed in rats and mice in vivo.

2. Results

2.1. Chemical structures of native FCSs and their modified derivatives

To obtain native FCSs with various regular sulfation patterns, FCSs of Mw of approximately 50–70 kDa, 1–6, were extracted and purified from six sea cucumbers by previously described methods [15]. Depolymerized FCSs (dFCSs) 7–12 (approximately 10–15 kDa) were the depolymerized fragments of 1–6 prepared using our own methods [32].

The chemical structures of 1–12 were characterized from monosaccharide compositions, functional groups, and NMR analyses (detailed data are presented in Supporting information S1) to indicate that they shared the structural features of FCSs. These have a chondroitin sulfate (CS)-like backbone and side chains of sulfated fucose (Fuc) units linked at the C-3 position of β-D-glucuronic acid (GlcA), but the sulfation patterns of α-fucose residues that were identified by 1D and 2D NMR (Figs. S1–S4) varied from one to another.

![Fig. 1. Structures of several glycosaminoglycans from mammalian, such as unfractionated heparin (UFH), dermatan sulfate (DS), chondroitin sulfate (CS), synthetic pentasaccharide sequence of heparin (Fondaparinux, Fdx) and fucosylated chondroitin sulfates from echinoderm. GlcA, Glucuronic acid; IdoA, Iduronic acid.](image-url)
As shown in Table 1, the percentage content of Fuc2S4S in fucose branches of these FCSs decreased from compound 1 to 6. Over 90% of the L-fucose in 1 was 2,4-di-O-sulfation (Fuc2S4S, type I), whereas sulfation patterns of 6 were major 3,4-di-O-sulfated fucose (Fuc3S4S, type IV). There was ~10% Fuc2S4S in compound 4 and hardly any in compound 5. In addition, more than half in 2 and 3 was Fuc2S4S; in 2, this included 4-O-sulfated fucose (Fuc4S, type III), whereas fucose residues in 3 also contained 3-O-sulfated fucose (Fuc3S, type II).

Almost all of the N-acetyl-b-D-galactosamine (GalNAc) in the CS-backbones of 1, 2, and 6 were 4,6-di-O-sulfated GalNAc (GalNAc4S6S). Apart from GalNAc4S6S, there was ~5% 4-O-sulfated GalNAc (GalNAc4S) in 3, and much more GalNAc4S and 6-O-sulfated GalNAc (GalNAc6S) present in 4 and 5, which could all be identified by 13C and 2D NMR analysis (Supporting information S1).

To the best of our knowledge, this represents the first report of the chemical structures of 1, from Stichopus monotuberculatus with 92% Fuc2S4S, and 6, from Holothuria scabra with ~85% Fuc3S4S (the fine chemical structural analysis of the two new compounds are shown in Supporting information S1). The structural distinctions between 2, from Apostichopus japonicas, and 3, from T. ananas, are consistent with previous reports [18,33]. Compound 4, from

### Table 1

Sulfate types and patterns of fucose residues in 1–12 and their anticoagulant activities.

<table>
<thead>
<tr>
<th>Compd. and resource</th>
<th>Mw (kDa)</th>
<th>Fuc type -SO3^- COOH</th>
<th>APTT (µg/ml)^b</th>
<th>TT</th>
<th>PT</th>
<th>Xase IIa/HCII</th>
<th>IIa/AT</th>
<th>Xa/AT (ng/ml)^g</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 S. monotuberculatus</td>
<td>53.7</td>
<td>92:1:24:18/1:0.4:4</td>
<td>2.50</td>
<td>10.0</td>
<td>/</td>
<td>14.4</td>
<td>497</td>
<td>202</td>
</tr>
<tr>
<td>2 A. japonicus</td>
<td>56.8</td>
<td>59:3/18:33</td>
<td>3.48</td>
<td>58.7</td>
<td>/</td>
<td>9.9</td>
<td>248</td>
<td>737</td>
</tr>
<tr>
<td>3 T. ananas</td>
<td>65.8</td>
<td>58:24:18:1</td>
<td>2.20</td>
<td>14.2</td>
<td>/</td>
<td>12.2</td>
<td>375</td>
<td>273</td>
</tr>
<tr>
<td>4 L. grisea</td>
<td>58.9</td>
<td>9:30:15:39</td>
<td>5.58</td>
<td>/</td>
<td>16.8</td>
<td>206</td>
<td>3660</td>
<td></td>
</tr>
<tr>
<td>5 H. nobilis</td>
<td>11.1</td>
<td>1:35:30:34</td>
<td>2.49</td>
<td>11.4</td>
<td>/</td>
<td>14.2</td>
<td>409</td>
<td>283</td>
</tr>
<tr>
<td>6 H. scabra</td>
<td>61.1</td>
<td>4:10:85</td>
<td>2.74</td>
<td>7.5</td>
<td>/</td>
<td>19.4</td>
<td>296</td>
<td>251</td>
</tr>
<tr>
<td>12 UFH</td>
<td>55.3</td>
<td>3:10:85</td>
<td>4.25</td>
<td>/</td>
<td>14.4</td>
<td>132</td>
<td>1794</td>
<td></td>
</tr>
<tr>
<td>14 LMWH</td>
<td>13.8</td>
<td>1:35:30:34</td>
<td>1.35</td>
<td>0.5</td>
<td>32</td>
<td>18.2</td>
<td>62</td>
<td>8.8</td>
</tr>
<tr>
<td>16 DS</td>
<td>41.4</td>
<td>4:10:85</td>
<td>1.35</td>
<td>0.5</td>
<td>32</td>
<td>18.2</td>
<td>62</td>
<td>8.8</td>
</tr>
<tr>
<td>18 OSCS</td>
<td>-18.0</td>
<td>1:35:30:34</td>
<td>7.80</td>
<td>4.0</td>
<td>/</td>
<td>48.8</td>
<td>184</td>
<td>49.1</td>
</tr>
</tbody>
</table>

a 1, Fuc2S4S; II, Fuc3S; III, Fuc4S; IV, Fuc3S4S.

b Molar ratio.
c Concentrations required to double the coagulant time.
d Anti-thrombin activities in the presence of AT.
e Anti-thrombin activities in the presence of AT.
f Anti-FXa activities in the presence of AT.
g Concentrations required 50% inhibition of factor’s activities.
h Lower than 1%.
i Above 1250 µg/ml.
j Above 10,000 ng/ml.
L. grisea, is similar to 5, from Holothuria nobilis, in terms of backbones and fucose branches, although the latter possesses less Fuc2S4S. Additionally, the chemical structure of 4 is basically in agreement with the literature, where the ratios of fucose branches were determined by analyzing products after mild acid hydrolysis [14]. In this work, the structure of 4 was identified from NMR spectra, for the consideration of sulfate loss during mild acid hydrolysis [18]. Overall, the chemical structures of native FCSs with various regular sulfation patterns have been clearly identified, suggesting that the six native compounds are representative of the abundant sulfate patterns of natural FCSs.

Depolymerized FCSs (13–21) of Mw ranging from 5 kDa to approximately 42 kDa (Scheme 1(a)) were obtained from depolymerization of 3 as in our previous description [31]. The physico-chemical properties of 13–21 were determined and compared with those of 3. The Mw distribution of 13–21 did not broaden significantly (Fig. S5); monosaccharide composition and specific rotations showed no significant variation during depolymerization (Table S2). The NMR spectra of 7–21 were all similar to those of the original compound 3 (Figs. S6–S8), implying that the other structural properties of these fractions were comparable. These results confirm the retention of the primary structure of 7–21 after depolymerization, which is consistent with our previous work [31].

Compound 22 was prepared from 16 by carboxyl group reduction, which was performed as previously described (Scheme 1(b)) [14]. Determinations by conductometric titration confirmed that the carboxyl groups of 22 were completely reduced. The esterified reaction was performed by the addition of an alkyl halide to an N,N-dimethylformamide solution of its ammonium salts (Scheme 1(c)–(e)) [33]. The esterified derivatives (23, 24, and 25) were synthesized from 16 and their degrees of esterification, determined by 1H NMR spectra, were 100%, 60%, and 40% for ethyl ester, benzyl ester and 1-butyl ester, respectively. Sulfated fucose branches, which were partially removed from 3 by mild acid hydrolysis provided 27, as described previously (Scheme 1(f))[14]. Compound 27 was prepared from 3 because mild acidic defucosylation was accompanied, to some degree, by depolymerization [18]. 1H NMR spectroscopy confirmed that ~64% of the fucose branches had been removed. Compound 26 was the partial N-deacetylated derivate of 16, which was prepared as described earlier (Scheme 1(g))[34]: the degree of deacetylation, confirmed by 1H NMR spectroscopy, was 49%. Compounds 22–27, resulting from the above procedures, were isolated as their sodium salts by precipitation in ethanol after prior addition of sodium chloride. Purification by gel filtration on a Sephadex G-25 column was performed to eliminate traces of contaminating mineral salts. Lyophilization yielded powders, which were easy to handle.

The compounds were characterized by 1H and 13C NMR spectroscopy. Typical chemical shifts for the newly introduced substituents of compounds 22–27 are shown in Fig. S9. Assignment of the 1H and 13C NMR signals of compounds 22–27 is described in Supporting Information S3. Conductometric titration determined that no desulfation occurred during the preparation of all the compounds. All of the products tested were readily soluble in water and, so, convenient to use in anticoagulant analysis in the present study.

2.2. Anticoagulant and anti-coagulant factor activities of compounds 1–21

The effects of native compounds 1–6 from six sea cucumbers with various regular sulfation patterns and their corresponding depolymerized products, 7–12, on anticoagulant activities are summarized in Table 1 (see also Fig. S10). The APTT-prolonging activities of 1–12 in human plasma were lower than those of UFH, but greater than those of LMWH, which indicated their potent inhibitory activity on the intrinsic pathway of blood coagulation. Compounds 1–3, with more than 50% Fuc2S4S, showed stronger anticoagulant activity than 4–6, whereas the activities of the depolymerized compounds 7–12, with Mw of approximately 11–15 kDa, were about 1.5–2-fold lower than those of the native compounds. The TT and PT assays indicated that concentrations required to double the TT of 1–6 were 2–5-fold higher than those required of LMWH, but 1–12 showed no observable effects on PT at the concentrations tested (<1.25 mg/ml).

Compounds 1–3 with higher contents of Fuc2S4S (>50%) were much stronger inhibitors of intrinsic Xase than UFH, and 4–6 were slightly less active inhibitors than UFH, but still more than 100% stronger than LMWH. Interestingly, the depolymerized compounds, 7–12, exhibited similar or stronger anti-Xase activities in comparison to the corresponding native compounds. Compounds 1–12 also displayed HCII-dependent antithrombin (anti-Xa) activity, which was weaker than that of UFH or LMWH. Interestingly, the HCII-dependent anti-Xa activities of the depolymerized compounds 7–12 were about 1.5–3 times stronger than those of the corresponding native compounds; 12, particularly, exhibited the strongest activity.

The AT-dependent anti-Xa activities of 1–12 were all much lower than those of LMWH or UFH, but compounds 1, 2, and 3 with a higher content of Fuc2S4S displayed about 1.5–2.5-fold stronger AT-dependent anti-Xa activities compared to their HCII-dependent anti-Xa activities in terms of IC50 values. The AT-dependent anti-Xa activity of 6 was similar to its HCII-dependent anti-Xa activity, while the activities of 4 and 5 were approximately one third and one fifth of their anti-Xa activities, respectively. This activity was significantly reduced down to between three and ten times less than that of the native FCSs after depolymerization. Additionally, compounds 1–12 exhibited much weaker AT-dependent anti-Xa activities than UFH and LMWH; in particular, 4–12 did not inhibit the activity of factor Xa by 50%, even at a concentration of 10 μg/ml. Overall, compounds 1–12 possessed very strong APTT-prolonging activity and directly inhibited factor Xa generation by the intrinsic tenase complex, and these compounds also exhibited, to an extent, AT- or HCII-dependent inhibition of thrombin. Among these compounds, 1–3, with more Fuc2S4S, exhibited stronger APTT-prolonging action and AT-dependent anti-Xa and anti-Xa activities, whereas 12, with more Fuc2S4S, showed the strongest HCII-dependent anti-Xa activity.

Meanwhile, the anticoagulant properties of several commercial products, such as UFH, LMWH, fondaparinux (Fdx), oversulfated chondroitin sulfate (OSCS), and DS, were also evaluated as controls. As expected, Fdx only showed strong AT-dependent inhibition of Xa, and OSCS exhibited strong activities apart from its weak effect on TT. DS displayed relatively strong HCII-dependent anti-Xa activity.

2.3. Anticoagulant properties of depolymerized compounds 13–21

As shown in Table 1, a comparison of the effects of 1–12 on anticoagulant factors suggests that the Mw of an FCS has an obvious influence on its activities. As native compound 3 possesses potent anticoagulant activity, various and well-defined sulfate patterns of fucose branches and backbones, and its structure is not as complicated as that of 4 and 5, 3 was chosen for the preparation, by chemically controlled depolymerization, of its fragments, 13–21, with gradually decreasing Mw.

The anticoagulant activities of native compound 3 and 13–21 were measured by APTT, PT, and TT assays in human plasma. The results of APTT assays indicated that Mw significantly affected anticoagulant activity (Table 2, see also Fig. S11). The concentrations required to double APTT increased significantly with
decreasing Mw. When APTT-prolonging activity was expressed as heparin U per mg using a parallel standard curve based on a heparin standard (212 units/mg), potencies decreased in proportion to the reduction in Mw, following a logarithmic-like function (activity corresponds to that of UFH). However, when Mw decreased further, Xase-inhibiting activities were markedly reduced (Table 2 and Fig. S1). These results demonstrated further that within a certain Mw range, the anti-Xase activity and HCII-dependent anti-IIa activities were markedly reduced (Table 2 and Fig. S1)

### Table 2
Anticoagulant properties of compounds 3, 13–21.

<table>
<thead>
<tr>
<th>Compd.</th>
<th>Mw (kDa)</th>
<th>APTT μg/ml</th>
<th>Xase U/mg</th>
<th>IIa/HCII ng/ml</th>
<th>IIa/AT ng/ml</th>
<th>Xa/AT ng/ml</th>
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<tr>
<td>3</td>
<td>65.8</td>
<td>4.29</td>
<td>112</td>
<td>13.3</td>
<td>290</td>
<td>483</td>
</tr>
<tr>
<td>13</td>
<td>42.6</td>
<td>4.87</td>
<td>99</td>
<td>12.1</td>
<td>319</td>
<td>421</td>
</tr>
<tr>
<td>14</td>
<td>23.4</td>
<td>6.09</td>
<td>79</td>
<td>11.5</td>
<td>336</td>
<td>299</td>
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<tr>
<td>15</td>
<td>17.2</td>
<td>6.27</td>
<td>77</td>
<td>10.8</td>
<td>357</td>
<td>284</td>
</tr>
<tr>
<td>16</td>
<td>14.0</td>
<td>6.64</td>
<td>72</td>
<td>11.9</td>
<td>324</td>
<td>242</td>
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<tr>
<td>17</td>
<td>12.7</td>
<td>7.20</td>
<td>67</td>
<td>11.4</td>
<td>338</td>
<td>200</td>
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<tr>
<td>18</td>
<td>11.6</td>
<td>7.50</td>
<td>64</td>
<td>11.7</td>
<td>329</td>
<td>163</td>
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<td>32</td>
<td>30.3</td>
<td>127</td>
<td>132</td>
</tr>
<tr>
<td>UFH</td>
<td>-18.0</td>
<td>2.27</td>
<td>212</td>
<td>18.2</td>
<td>212</td>
<td>67</td>
</tr>
<tr>
<td>LMWH</td>
<td>-4.50</td>
<td>9.32</td>
<td>52</td>
<td>52.7</td>
<td>73</td>
<td>188</td>
</tr>
<tr>
<td>DS</td>
<td>41.4</td>
<td>47.7</td>
<td>10</td>
<td>&gt;2000</td>
<td>&lt;2</td>
<td>98</td>
</tr>
</tbody>
</table>

* a The average molecular masses (Mw) of compounds 3, 13–21 were determined by HPGC–LALLS.
  b The concentration required to double the APTT of human plasma (APTT doubling).
  c Expressed as heparin units/mg using a parallel standard curve based on a heparin standard (212 units/mg).
  d IC90 value, the concentration required to inhibit 50% of protease activity.
  e Above 10,000 ng/ml.

### 2.4. Anticoagulant activities of functionally modified compounds 22–27

Compound 16 was selected for the preparation of chemically modified derivatives with potent anti-Xase and HCII-dependent anti-IIa activities. The structures of 22–27 were also well defined from the analysis of their physicochemical characteristics and NMR spectra. The results indicate that the basic chemical structures of depolymerized and modified products did not change measurably (Supporting information S2 and S3), except that the Mw decreased or the groups were modified.

As shown in Fig. 5 (see also Table S3), in comparison to 16, the APTT-prolonging activity of 22 decreased by about 74% following carboxyl reduction, whereas the activities of 23 with ethyl ester, 24 with benzyl ester, 25 with 1-butenyl ester and deacetylated compound 26, were also reduced slightly by 69%, 48%, 33% and 32%, respectively. It is worth noting that the APTT-prolonging activity of 27 was reduced down to ~15% of that of 16.

Modifications of functional groups also affected anti-Xase activities. The inhibitory potency of carboxyl-reduced compound 22 was equivalent to only 24% of that of 16, whereas the activities of 23 (ethyl ester), 24 (benzyl ester), and 25 (1-butenyl ester) were reduced by 53%, 65%, and 4% of 16, respectively. The modifications producing the least and greatest effects were deacetylation and defucosylation, with the activities of 26 and 27 equivalent to 119% and 2.6% of that of 16, respectively.

The HCII-dependent anti-IIa activities of 22, 23, 25, and 26 were similar to that of 16, but that of 24 decreased to 2.5 times that of 16. The intriguing finding was that partial defucosylation did not decrease the HCII-dependent anti-IIa activities of 27; this modification increased the activity of 27 to ~3 times that of 16.

### 2.5. Factor XII and platelet activation

It has been reported that FCS from L. grisea activates factor XII and causes hypotension when injected intravenously into rats [24,29]. Therefore, the effects of 1–26 on the activation of factor XII were investigated.

Compounds 1–6 with various sulfation patterns were demonstrated to induce significantly factor XII activation (Fig. 6A). Factor XII activation activity of depolymerized products, 7–12, was markedly weaker than that of the native compounds at a concentration of less than ~5 μg/ml, but activities were still strong at higher concentrations. However, LMWH activated factor XII slightly at a concentration of less than 60 μg/ml (Fig. 6A).

Notably, comparisons between the activation of factor XII by 3 and by its depolymerized compounds, 13–21, suggested that activity was increased from Mw, especially at concentrations of lower than 30 μg/ml (Fig. 6B). When Mw was reduced to below 12 kDa (19–21), factor XII activation was weakened to close to, or below, that of UFH. In addition, some compounds with chemical modifications, such as carboxyl reduction (22), esterification (23–25), and N-deacetylation (26), could also reduce the activation of factor XII at low concentrations, though activity remained strong as concentrations increased (Fig. 6C).

The effects of these compounds on platelet aggregation were also studied using a conventional turbidimetric assay. Aggregation was quantified by measuring the maximum extent of the increase
in light transmittance 8 min after addition of the agonist. Native compound 3 from T. ananas (other native FCSs, the same as 3, not shown) caused platelets to aggregate significantly in citrated human PRP (15–30 mg/ml), in a similar manner to ADP, the positive control (Fig. 7). This result is consistent with observations of native FCS from A. japonicas [28].

However, platelet activation by FCS could be reduced dramatically by decreasing Mw (Fig. 7A and B). In particular, at a Mw below 12 kDa, these compounds did not induce platelets to aggregate significantly, even at a concentration of 30 μg/ml, which was activity as weak as that of UFH. Additionally, chemically modified products (Mw < 12 kDa) did not produce observable profiles on human platelet aggregation at the two concentrations tested (Fig. 7C and D). These results suggest that platelet activation by FCS may be roughly dependent on their molecular size.

2.6. Antithrombotic activity and effects on bleeding in vivo

Our results indicate that the inhibition of intrinsic factor Xase displayed by compounds 16–19, with Mw of 8–14 kDa, was still as strong as that of native compound 3 (Figs. 2–4), while the ability to activate factor XII and platelets significantly decreased (Figs. 6 and 7). Therefore, it was worthwhile determining the antithrombotic action of a 6–12 kDa fragment of 3 in vivo (for example, 19, with Mw~8.5 kDa).

In the phospholipid-kaolin-induced venous thrombosis model, which was activated by the intrinsic coagulation pathway, 19 displayed much stronger inhibition of venous thrombus formation at a dose of 14.8 mg/kg (p < 0.001); this was similar to LMWH at a dose of 14.4 mg/kg (Fig. 8A). Interestingly, 19 showed much stronger activity than native compound 3 at the same dose (thrombosis inhibition of 85% vs. 35%).

To evaluate the effect of sulfated polysaccharides on bleeding, we used the bleeding assay. In this model (Fig. 8B), LMWH significantly increased blood loss at doses of 21, 42, and 83 mg/kg body weight, which were about 1.5–, 3–, and 6-fold, respectively, of the dose required for the inhibition of venous thrombosis. In contrast,
antithrombotic is severely limited due to its undesirable effects, such as factor XII and platelet activation [24,25,28,29]. The anticoagulant mechanism of heparin is mainly an AT-dependent pathway, which requires a high-affinity pentasaccharide with a special saccharide sequence and sulfate pattern [4]. The pentasaccharide sequence alone will accelerate the inactivation of factor Xa by AT, whereas a longer sequence (≥18 sugar units, including the pentasaccharide) is required for the inactivation of thrombin [5]. In the present study, in order to clarify whether the structural features and chain length of FCSs affect the potency and selectivity of their inhibition of factor Xase, the effects of sulfate pattern, molecular weight, and chemical modification of these compounds on anticoagulant activity and on the inhibition of several coagulant factors were investigated. Moreover, the anticoagulant, antithrombotic, and effects on bleeding were evaluated by administering modified compounds subcutaneously to rats and mice *in vivo*.

In the present work, we have investigated structure–activity relationships of a series of FCS derivatives. The results of this study indicate that 1–3 with more Fuc2S4S, displayed stronger APTT-prolonging, AT-dependent anti-IIa, and anti-Xa activities, which suggests that the occurrence and percentage content of Fuc2S4S may play a critical role in the anticoagulant activities of FCS compounds [24,30]. In contrast, 4–6 with more Fuc3S4S, exhibited stronger HCII-dependent anti-IIa activities. Additionally, all of these native compounds with various sulfation patterns of fucosyl-containing side chain have potent anti-Xase activities. However, to determine whether the inhibition of Xase requires a special sulfation pattern or saccharide sequence requires further study.

In several preliminary research studies, the anticoagulant activities and HCII-dependent activities of subfractions of FCS from *L. grisea* and depolymerized FCS from *A. japonicas* with lower Mw were relatively weak compared to corresponding native ones [17,22]. In the present study, when compared with the corresponding native FCSs, the APTT-prolonging activities of depolymerized compounds 7–12 decreased, but notably, their anti-Xase and HCII-dependent anti-IIa activities were very similar or stronger than those of the native compounds. This led us to further study the activity of FCS fragments of different Mw.

The pattern of the effects of Mw on above anticoagulant activities were not the same (Table 1, Figs. 2–4), as was observed, for the heparin fractions [35]. The APTT-prolonging activity of 13–21

3. Discussion and conclusions

Since intrinsic factor Xase is the last target enzyme for inhibition of the intrinsic coagulation pathway [9,10], it is becoming recognized as a prime target for developing safer anticoagulants with potential physiological and therapeutic applications [21]. FCS, a structurally unusual sulfated glycosaminoglycan [13–15], has potent factor Xase inhibitory activity, which makes it of special interest as a putative drug [16,18,20]. However, the application of FCS as a novel
decreased in proportion to the reduction in Mw, which is consistent with our previous observations [32]. The anti-Xase activity of depolymerized FCSs did not decrease at a Mw over ~8.5 kDa, while activity rapidly decreased as Mw was further decreased, suggesting that there may be a threshold chain length of 9–10 backbone disaccharide units required for FCS to exhibit inhibition of factor Xase fully.

Clearly, as factor Xase and HCII-dependent IIa inhibition by fragments of Mw higher than ~8.5 kDa was not less than that of native compounds, weakening of the APTT-prolonging activities with decreasing Mw may mostly contribute to the reduced AT-dependent anti-IIa activities. However, over a Mw of ~8.5 kDa, the reduction in the APTT-prolonging activities of FCS fragments may mostly result from a decrease in anti-factor Xase activity because AT-dependent anti-IIa activity is likely to be too weak to affect the APTT-prolonging action significantly (Fig. S11).

A reduction in the carboxyl groups of GlcA in FCS obtained from L. grisea does not affect its anticoagulant or antithrombotic actions, but partial defucosylation abolishes them [14,23,36]. In the present study, our data suggest that a free carboxyl of GlcA may be required for FCSs to demonstrate anti-Xase activity. Partial defucosylation caused a sharp decrease in its APTT-prolonging and anti-Xase activities. However, HCII-dependent anti-IIa activities were not affected in the same way. These results suggest that fully fucosylated GlcA may account for potent APTT-prolonging and anti-Xase activities.

Fig. 6. Activation of factor XII in the presence of compounds, UFH, LMWH, and OSCS. Standard human plasma was diluted with three volumes of TS buffer (0.02 M Tris/HCl, 0.15 M NaCl, pH 7.4). The plasma sample (30 μl) and a 30 μl glycosaminoglycan solution were incubated at 37 °C for 2 min. Then, 30 μl of 6 mM kallikrein chromogenic substrate CS-31(02) was added and the absorbance at 405 nm was recorded for 450 s. Results are shown as means of duplicates.

Fig. 7. Profile of the platelet aggregation induced by compounds 3, 13–21 (A, B) and 22–27 (C, D). The platelet activation were performed at 15 μg/ml (A, C) and 30 μg/ml (B, D) of concentrations of these compounds in comparison to ADP (10 μM), UFH (30 μg/ml).
activities, while partial fucosylation may account almost totally for the potent HCII-dependent anti-IIa activities.

Factor XII is known to be a component of the coagulation system, which can be activated by contact with polyanionic material, such as OSCS [37], dextran sulfate [38], and heparin [39]. It has been reported that FCS from L. grisea can also induce XII activation [24,29]. Our data revealed that native FCSs display a strong ability to activate factor XII, and Mw has a significant impact on this. As the Mw of FCSs dropped to approximately 6–12 kDa, activity was found to be close to, or lower than, that of heparin. With respect to a further undesirable effect of native FCSs, a decrease in Mw dramatically reduced platelet activation. At a Mw of 12 kDa or below, these compounds displayed similarly weak platelet activation activity to UFH.

In summary, different types of sulfated fucose branches, various saccharide chain lengths, and the modification of different functional groups of FCSs resulted in compounds with distinct effects on anticoagulant and coagulant factors. This might allow the selection of FCSs, or their derivatives, with strong inhibitory activity of human intrinsic Xase, but with effects on AT and factor XII activation, which are as low as possible. In comparison with the anticoagulant activities of 1–27, we found that 19, of ~8.5 kDa, is a strong anti-factor Xase agent, but has very weak AT-dependent anti-IIa activity and activation of factor XII, and is devoid of inducing platelet aggregation. Therefore, it was of significant interest to assess the antithrombotic action of this compound in vivo.

When injected subcutaneously into rats, 19 displays the same antithrombotic activity as LMWH in thrombin-induced venous thrombosis models. LMWH has stronger antithrombotic activity via the AT-dependent anticoagulant mechanism [40,41], but notably antithrombotic activity of 19 may mostly be mediated by the inhibition of factor Xa generation by the intrinsic tenase complex. Because 19 exhibits strong inhibition of factor Xa generation and LMWH inhibits factor Xa, it may be easy to relate the antithrombotic activities to a corresponding mechanism. Additionally, our results indicate that 19 displayed significantly weaker platelet activation than 3 (Fig. 7). Hence, it is reasonable that the greater potency of 19 over 3 at inhibiting venous thrombosis may partially be correlated with its ability to reduce platelet activation.

An ideal anticoagulant agent for clinical use should have specific antithrombotic activity without increasing the risk of bleeding [41]. Literature surveys revealed that inhibitors of the coagulant factors in the intrinsic pathway should effectively prevent thrombus formation, with a negligible risk of bleeding [7,8]. As the antithrombotic mechanism of depolymerized FCS may principally involve the inhibition of Xase, the cutting mouse-tail bleeding model was used to assess the hemorrhagic effect of 19. The results showed that, when compared with LMWH at a similar dose, blood loss after administration of 19 was lower (Fig. 8B).

In clinical studies, LMWH displays superior pharmacokinetic properties to UFH, and the risk of bleeding was much lower compared to that with UFH, when antithrombin activity was reduced relative to anti-Xa activity [41]. Our results demonstrate that in rats, 19 possesses greater antithrombotic activity than 3 (Fig. 8A) without increasing the risk of bleeding (Fig. 8B). This may be due to the specific selectivity of 19 for coagulant factors.

In conclusion, the present study has demonstrated that the Mw and structural features of FCS affect the anticoagulant potency and its selectivity for coagulant factors. Our results also suggest that depolymerized fragments from 3 with Mw of approximately 6–12 kDa possess strong anti-factor Xase activity, reduce AT-dependent antithrombin action, and have negligible factor XII and platelet activation activities. For example, the subcutaneous administration of 19, of ~8.5 kDa, inhibits venous thromboses induced by the activation of the intrinsic pathway in animals, with little or no risk of excessive bleeding relative to LMWH. Hence, the data presented support further experimentation on the low Mw compounds as they represent potential antithrombotic therapeutic agents for the selective inhibition of factor Xa generation by the intrinsic tenase complex.

4. Experimental procedures

4.1. Materials

UFH (212 USP U/mg) and dermatan sulfate (DS) (~41,400 Da) were purchased from Sigma (USA). LMWH (Enoxaparin, 0.4 ml × 4000 AXaIU) was from Sanofi-Aventis (France). Over-sulfated chondroitin sulfate (OSCS) was obtained from Serva Electrophoresis GmbH (Germany). The activated partial thromboplastin time (APTT), prothrombin time (PT), thrombin time (TT) reagents,
and standard human plasma were from Teco Medical (Germany). Biophen FVIII: C kit, Biophen Heparin Anti-IIa kits, Biophen Anti-thrombin 2.5 kits, Human HClII, AT, thrombin, prekallikrein, human factor Xla, thrombin chromogenic substrate CS-01(38), kallikrein chromogenic substrate CS-31(02), and factor Xa chromogenic substrate Sxa-11 were all from Hyphen Biomed (France). Human factor VIII was from Green Cross China, Inc. (China). Human factor XII was purchased from Assaypro (USA). Bovine thrombin and kaolin were obtained from Sigma (USA). ADP was purchased from the Chronolog Corporation (USA). All other chemicals were of reagent grade and obtained commercially.

4.2. General procedure for compounds 1–21

Native FCSs 1–6, from the body walls of six sea cucumbers: S. monotuberculatus, A. japonicus, T. ananas, L. grisea, H. nobilis, and H. scabra were extracted, prepared, and purified as previously described [14,15]. Yields were approximately 1.0–1.5%. Six depolymerized fragments of 1–6 were obtained by controlled chemical depolymerization as described previously [31], and were designated as compounds 7–12, respectively. Similarly, nine depolymerized fragments of 3 were obtained and designated as compounds 13–21, respectively. The chemical shifts in Table S1 were based on interpretations of correlated spectroscopy (COSY), total correlation spectroscopy (TOCSY), rotating frame overhauser effect spectroscopy (ROESY), and 1H/13C heteronuclear single quantum coherence (HSQC), heteronuclear multiple bond coherence (HMBC) spectra of compounds 7–12 (Supporting information S1). The Mw of compounds 13–21 were examined by high-performance gel permeation chromatography—low–angle light scattering (HPGPC), as described previously [32], and are shown in detail in Supporting information S2. Chemical compositions, intrinsic viscosity, and specific optical rotation of compounds 3 and 13–21 are also shown in the Supporting information S2. The NMR spectra for compounds 13–21, including the 1H, 13C, COSY, TOCSY, ROESY, HMBC, and HSQC spectra, were similar to those of compound 3 in our previous study (see Supporting information S2) [31].

4.3. General procedure for compound 22

Reduction of the carboxyl groups in compound 16 was performed as described by Mourão et al. [14]. About 100 mg of compound 16 was dissolved in 16 ml water, and the pH of the solution was adjusted to 4.75 with 0.1 M HCl. Solid L-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC, 300 mg) was added over a period of 5 min, and the pH was maintained at 4.75 with 0.1 M HCl. About 1.2 g of solid NaBH4 was slowly added with stirring, the solution was heated to 50 °C and maintained at this temperature for 2 h. After addition of several drops of acetic acid to destroy the excess of borohydride, the solution was dialyzed (cut-off 3.5 KD) against distilled water and lyophilized. About 80 mg (80% yield) of carboxyl-reduction product (compound 22) was obtained as a white solid. A conductometric titration assay confirmed that the carboxyl groups of compound 22 were completely reduced. \([\Delta ]_D = 56.4^\circ (c = 1.0, H_2O)\). 1H NMR (D2O, 500 MHz): 0.43 (3H, Gc), 3.56 (H2, Gc), 3.74 (H3, Gc), 3.83 (H4, Gc), 3.38 (H5, Gc), 3.61/3.84 (H6, Glc).

4.4. General procedure for compounds 23–25

Esterification products were synthesized as previously described [42,43]. The benzyl ester derivative, 23, was synthesized as follows: ethyl bromide (0.16 ml) was added to an N,N-dimethylformamide (2.5 ml) solution of the tetrabutylammonium salts of compound 16 (240 mg) and the solution was maintained at 35 °C for 24 h. Aqueous 0.5 M NaCl (2.5 ml) and absolute alcohol (25 ml) were added. The precipitate was collected, dissolved in deionized water, and converted to the sodium salt via Dowex-50 (H+) ion exchange and NaOH neutralization. Compound 23 was obtained after lyophilization (175 mg, 73%). Similarly, compounds 24 and 25 were synthesized as described for 23, using the appropriate alkyl halide. 1H NMR and conductometric titration analysis showed that the degrees of esterification were 100%, 60%, and 40% for the ethyl ester, benzyl ester, and 1-butenyl ester, respectively. 1H NMR (D2O, 500 MHz) of compound 23: 0.48 (CH3CH2), 1.32 (CH2CH3), compound 24: 0.47–0.59 (m, 5H, Ph), 4.70/4.51 (J = 11.4, PhCH2); compound 25: 0.252 (CH3CH2CH2), 4.30 (CH2CH2CH2), 5.05–5.20 (CH2CH2CH2), 5.85 (CH2CH2CH2).

4.5. General procedure for compound 26

Compound 26 was the derivative of compound 16 after N-deacetylation [34]. To obtain compound 26, about 250 mg of compound 16 was placed in a tube; the reaction was carried out at 90 °C in hydrazine (6 ml) containing 1% hydrazine sulfate (60 mg) for 10 h under vacuum. The solution was dialyzed and lyophilized to give 196 mg product (78.4% yield). 1H NMR confirmed that the degree of deacetylation was ~49%. The chemical shifts assigned are shown elsewhere [34].

4.6. General procedure for compound 27

Compound 27 was the chondroitin sulfate backbone, obtained by mild acid hydrolysis of 3 [14]. About 200 mg of 3 was subjected to partial acid hydrolysis in 40 ml 100 mM H2SO4 at 100 °C for 60 min. The pH of the solution was adjusted to 7.0 with 1 M NaOH. Absolute alcohol (150 ml) was added and the free fucoses were removed by centrifugation. The precipitate was collected, dissolved in deionized water, dialyzed, and lyophilized. About 115 mg (57.5% yield) of compound 27 was obtained as a white solid. The degree of defucosylation was 64%, as determined by 1H NMR. The chemical shifts of compound 27 have been assigned and reported in a previous publication [18].

4.7. Chemical characteristics and structural analysis

The total hexose, methylpentose, hexuronic, and hexosamine in compounds 1–16 were determined as previously described [44–46]. The ratio of GalNAc and Fuc was obtained from 1H NMR analysis integrals of their methyl protons. The sulfate/carboxyl groups were determined with a conductometric method [47]. The specific optical rotation of 10 ml of 1% (w/v) samples in water was determined at 25 °C.

Structural analysis of all samples was performed by NMR analyses at 60 °C or 27 °C in distilled water with a Bruker Avance spectrometer equipped with a 13C/1H dual probe in FT mode, as previously described [48], at 500 or 600 MHz. All spectra were recorded with HOD suppression by presaturation. 1H–1H COSY, TOCSY, NOESY, 1H/13C HSQC, and HMBC spectra were recorded using state-time proportional phase incrementation for quadrature detection in the indirect dimension. All chemical shifts were relative to internal trimethylsilyl-propionic acid (TSP).

4.8. Determination of anticoagulant activities in vitro

APTT, PT, and TT were determined with a coagulometer (TECO MC-4000, Germany) using APTT, PT and TT reagents and standard human plasma as previously described [49].
4.10. Inhibition of human thrombin by HCII

Inhibition of thrombin by human HCII was measured with thrombin chromogenic substrate, CS-01(38), using a previously described method with modifications [21,34]. A mixture containing 30 μl of HCII (1 μM) and 30 μl of various amounts of each glycosaminoglycan was incubated at 37 °C for 2 min. A 30 μl aliquot of 20 NIH/ml thrombin was then added. After incubation at 37 °C for 1 min, 30 μl of 4.5 mM thrombin chromogenic substrate CS-01(38) solution was added and the thrombin activity was measured. Absorbance at 405 nm was measured.

4.11. Inhibition of human thrombin and factor Xa by AT

Antithrombin and anti-factor Xa activities in the presence of AT were measured with Biophen Heparin Anti-IIa kits and Biophen Antithrombin 2.5 kits. A mixture of 30 μl samples and 30 μl 1 IU/ml AT was incubated at 37 °C for 2 min; 30 μl of 24 NIH/ml thrombin (or 8 μg/ml bovine factor Xa) was added. After incubation for 2 min (or 1 min for factor Xa), the residual thrombin or factor Xa activity was measured by the addition of 30 μl of 1.25 mM thrombin chromogenic substrate CS-01(38) or 1.2 mM factor Xa chromogenic substrate SXa-11. The absorbance of the reaction mixture was read at 405 nm.

4.12. Activation of human factor XII

The activation of human factor XII in the presence of glycosaminoglycans was determined by a previously described method with modifications [29]. Standard human plasma was diluted with three times the volume of TS buffer (0.02 M Tris/HCl, 0.15 M NaCl, pH 7.4). The plasma sample (30 μl) and a glycosaminoglycan solution (30 μl) were incubated at 37 °C for 2 min. Then, 30 μl of 6 mM CS-31(02) was added and the absorbance at 405 nm was recorded for 450 s. CS-31(02) is a chromogenic substrate for plasma kallikrein, which is activated from its precursor, prekallikrein by the action of factor XIIa.

4.13. Antithrombotic properties and bleeding effects in vivo

4.13.1. Animals

Sprague Dawley rats (of body mass 250–300 g) and Kunming mice (of body mass 18–22 g) from Sichuan Academy of Medical Sciences (Chengdu, PR China) and Vital River Laboratory Animal Technology Co., Ltd (Beijing, PR China) were used for animal experiments. Rats and mice were caged and fed a regular diet for at least 1 week before use. These experiments were reviewed and approved by the Animal Ethics Committee of Kunming Institute of Botany, Chinese Academy of Sciences.

4.13.2. Stasis-induced venous thrombosis

Thrombus formation was induced by promoting a combination of stasis and hypercoagulability using a modification of the method of Vogel et al. [50]. Vehicle, compound 3, or 19, or LMWH were administered dorsally and subcutaneously. After 60 min, rats were anesthetized by 10% chloral hydrate (0.3 ml/kg), and two loose sutures were prepared 0.7 cm apart on the inferior vena cava and all the collateral veins were ligated. Phospholipid-kaolin mixed liquor (4%) was injected intravenously. At a time of 20 s following the injection, stasis was established by tightening the two sutures: the proximal and then the distal. The abdominal cavity was provisionally closed and stasis was maintained for 20 min. The cavity was then reopened, the ligated segment was opened longitudinally, and the thrombus formed was removed, rinsed, blotted on filter paper, dried for 24 h at 50 °C, and weighed. For each group (n = 10), the mean thrombus mass was determined and then expressed as a percentage of thrombus mass. The absence of any inhibition of thrombus formation (compared to the mean thrombus mass in the group administered vehicle) was represented as 100%.

4.14. Statistical analysis

The results of the coagulation assays were calculated as sigmoid or linear regressions using an Origin 8.0 progress function (OriginLab, USA), according to a method described previously [11]. The results of the venous thrombosis and bleeding effect experiments were expressed as means ± SDs and were analyzed by SPSS statistics version 16.0 (IBM, USA), and the statistical significance of results was determined using one-way analysis of variance (ANOVA), followed by Levene’s test or the chi-square test. Data were considered significantly different when: *p < 0.05, **p < 0.01 or ***p < 0.001.
Conflict of interest
The authors state no conflict of interest.

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Appendix A. Supplementary data
Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2014.12.054.

References
[29] L.Y. Zhao, S.S. Lai, R. Huang, M.Y. Wu, N. Gao, L. Xu, H.B. Qin, W.L. Peng,


