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Novel chemo-enzymatic oligomers of cinnamic acids as direct and indirect inhibitors of coagulation proteinases

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Abstract—Thrombin and factor Xa, two important procoagulant enzymes, have been prime targets for regulation of clotting through the direct and indirect mechanism of inhibition. Our efforts on exploiting the indirect mechanism led us to study a carboxylic acid-based scaffold, which displayed major acceleration in the inhibition of these enzymes [*J. Med. Chem.* **2005**, *48*, 1269, 5360]. This work advances the study to chemo-enzymatically prepared oligomers of 4-hydroxycinnamic acids, DHPs, which display interesting anticoagulant properties. Oligomers, ranging in size from tetramers to pentadecamers, were prepared through peroxidase-catalyzed oxi\dative coupling of caffeic, ferulic, and sinapic acids, and sulfated using triethylamine–sulfur trioxide complex. Chromatographic, spectroscopic, and elemental studies suggest that the DHPs are heterogeneous, polydisperse preparations composed of inter-monomer linkages similar to those found in natural lignins. Measurement of activated thromboplastin and prothrombin time indicates that both the sulfated and unsulfated derivatives of the DHPs display anticoagulant activity, which is dramatically higher than that of the reference polyacrylic acids. More interestingly, this activity approaches that of low-molecular-weight heparin with the sulfated derivative showing ~2- to 3-fold greater potency than the unsulfated parent. Studies on the inhibition of factor Xa and thrombin indicate that the oligomers exert their anticoagulant effect through both direct and indirect inhibition mechanisms. This dual inhibition property of 4-hydroxycinnamic acid-based DHP oligomers is the first example in inhibitors of coagulation. This work puts forward a novel, non-heparin structure, which may be exploited for the design of potent, dual action inhibitors of coagulation through combinatorial virtual screening on a library of DHP oligomers.

1. Introduction

Thrombin and factor Xa, two critical enzymes of the coagulation cascade, have been targets of anticoagulation drug design for a long time.[1–4] Both enzymes can be inhibited directly or indirectly. Traditional anticoagulants, including heparin, low-molecular-weight heparin (LMWH) and warfarin, are indirect inhibitors, which mediate their effects through an intermediary co-factor, such as antithrombin or vitamin K. For the past 7 decades these indirect inhibitors have been the mainstay of anticoagulant therapy. Yet, they suffer from several limitations, such as enhanced bleeding risk, unpredictable response, heparin-induced thrombocytopenia, and lack of inhibition of clot-bound thrombin.

In contrast to indirect inhibition, direct inhibition of thrombin and factor Xa has been thought to be a better alternative, which promises to offer the important

Abbreviations: AT, antithrombin; APTT, activated partial thromboplastin time; CA, caffeic acid; CD, dehydropolymer of caffeic acid; CD_{AC}, acetylated dehydropolymer of caffeic acid; CD_S, sulfated dehydropolymer of caffeic acid; (+)-CS, (+)-catechin sulfate; DHP, dehydrogenation polymer; FA, ferulic acid; FD, dehydropolymer of ferulic acid; FD_{AC}, acetylated dehydropolymer of ferulic acid; FD_S, sulfated dehydropolymer of ferulic acid; HRP, horseradish peroxidase; IC_{50}, concentration of inhibitor that results in 50% inhibition; LMWH, low-molecular-weight heparin; MES, 2-(*N*-morpholino)ethanesulfonic acid sodium; M_N, number average molecular weights; M_W, weight-average molecular weights; PEG, polyethylene glycol; PT, prothrombin time; SA, sinapic acid; SD, dehydropolymer of sinapic acid; SD_{AC}, acetylated dehydropolymer of sinapic acid; SD_S, sulfated dehydropolymer of sinapic acid; SEC, size-exclusion chromatography; THF, tetrahydrofuran.

Keywords: Anticoagulant agents; Inhibition; Thrombin; Factor Xa; Dehydropolymers; Synthetic Lignins; Heparins.

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advantage of inhibition of both circulating and clot-bound thrombin. A prototypic member in this class of inhibitors is hirudin, which targets the active-site and exosite I of thrombin, and several derivatives of this peptide are now clinically available.[5,6] Intensive efforts are also being made to develop the first orally bio-available direct thrombin inhibitor. These are small molecule pro-drugs that target the active site of these enzymes.[7,8] However, challenges with these molecules include establishing enzyme binding affinity that is not associated with excessive bleeding, achieving inhibition of both clot-bound and unbound proteinases, and avoiding liver toxicity.[3,6]

The traditional anticoagulant, heparin or LMWH, is an anticoagulant of choice because of its good efficacy and easy availability. It is a linear co-polymer of glucosamine (GlcN*p*) and iduronic acid (IdoA*p*) residues linked in a $1 \rightarrow 4$ manner.[9,10] Yet, heparin is a complex, heterogeneous, polydisperse molecule. Further, the high sulfation level of heparin generates massive negative charge density, thereby introducing an ability to bind to a large number of proteins in the plasma,[11] a probable cause for some of its side-effects.

To reduce the limitations of heparin therapy, we have focused on designing scaffolds that possess much lower anionic character, have more hydrophobic nature, and yet retain the function of heparin.[12–16] In the process, we have designed some small sulfated flavonoids that utilize the antithrombin conformational activation mechanism for factor Xa inhibition[12–14] and have studied polymers of acrylic acid that utilize the bridging mechanism for inhibiting thrombin.[15,16] While our sulfated flavonoids displayed some 20-fold acceleration, polyacrylic acids (PAAs) displayed a massive 300- to 1100-fold acceleration in antithrombin-dependent inhibition of factor Xa and thrombin. Yet, PAAs were not likely to be useful as anticoagulants because of their poor antithrombin binding affinity and ability to chelate Ca^{2+} ions under physiological conditions.[16] We reasoned that both these problems could be addressed simultaneously through the introduction of two features—a more rigid hydrophobic backbone and some sulfate groups—in the PAA scaffold.

Scaffolds of the size of heparin (or LMWH) that simultaneously possess these two properties are difficult to find. Other than natural polysaccharides, only a couple of natural macromolecular structures are known—condensed tannins and lignins—that could possibly be simultaneously hydrophobic and anionic. Whereas condensed tannins, or proanthocyanidins,[17,18] are polymers of either flavan-3-ol, or flavan-4-ol, or flavan-3,4-diol monomers, lignins are polymers of phenylpropanoid monomers.[19,20] The presence of aromatic skeleton and phenolic, as well alcoholic, groups in both these structures ensures a combination of hydrophobic and anionic character.

We chose to explore lignin derivatives in view of the considerable literature available on the chemo-enzymatic synthesis of lignins.[21–25] Synthetic lignins, also called dehydrogenation polymers (DHPs), are prepared from cinnamyl alcohol monomers using oxidative radical coupling and possess at least four types of inter-monomer linkages, including β-*O*-4 and β-5 (Fig. 1). These variations introduce significant heterogeneity and complexity in the macromolecule, thereby generating high structural diversity necessary for rapid evaluation of structures.

We describe our initial results with oligomers of cinnamic acid as novel coagulation inhibitors. The DHP oligomers were prepared in good yields through chemo-enzymatic oxidative coupling of 4-hydroxycinnamic acids.[22,23,25] The DHPs prolong activated thromboplastin and prothrombin time, APTT and PT, respectively, with approximately equal potency as LMWH. Preliminary studies suggest that the DHPs inhibit factor Xa and thrombin in an antithrombin—dependent and—independent manner suggesting an interesting dual inhibition approach. While in depth studies are necessary to understand specific structural and mechanistic aspects of these DHPs, our results put forward potent, non-heparin structures, the 'lignin carboxylates', for rational, anticoagulant drug design.

2. Results

2.1. Synthesis of dehydrogenation polymers

Three cinnamic acid derivatives, caffeic acid, ferulic acid, and sinapic acid, were chosen for homo-polymerization primarily due to their ability to hydrogen bond, form ionic interactions, and undergo sulfation. HRP–catalyzed oxidation of these monomers generates radical intermediates, especially I_1 and I_2 (Fig. 1), which couple with monomers to give β-*O*-4- and β-5-linked dimeric units. These units undergo chain extension with radicals, such as I_1 and I_2, to give DHPs. Simultaneously, side reactions, such as decarboxylation, may occur to give variant oligomers. The DHPs were judged to be heterogeneous through size-exclusion and reverse-phase chromatographies (not shown).

To determine the average molecular weight of these polymers, we synthesized their acetylated derivatives CD_{AC}, FD_{AC} and SD_{AC}, and utilized non-aqueous SEC, a technique found useful for lignins and cinnamyl alcohol-based DHPs.[24] CD_{AC} and SD_{AC} gave comparable SEC chromatograms, while FD_{AC} displayed a significant shift toward higher molecular weight species (Fig. 3). The peak-average molecular weight (M_P) of acetylated CD, FD, and SD was found to be 1180, 2480, and 1190 Da, respectively, while the number-average molecular weight (M_N) was 880, 1870, and 1020 Da, respectively, suggesting unsymmetrical distribution of higher and lower molecular weight chains. The weight-average molecular weight (M_W) was found to be 2800, 3650, and 2990 Da, respectively, indicating that on average these oligomers are reasonably similar. Yet, the proportion of smaller chains is higher for both CD_{AC} and SD_{AC} than for FD_{AC}. Using the molecular weight of acetylated monomers, the average oligomer is estimated to be between 4

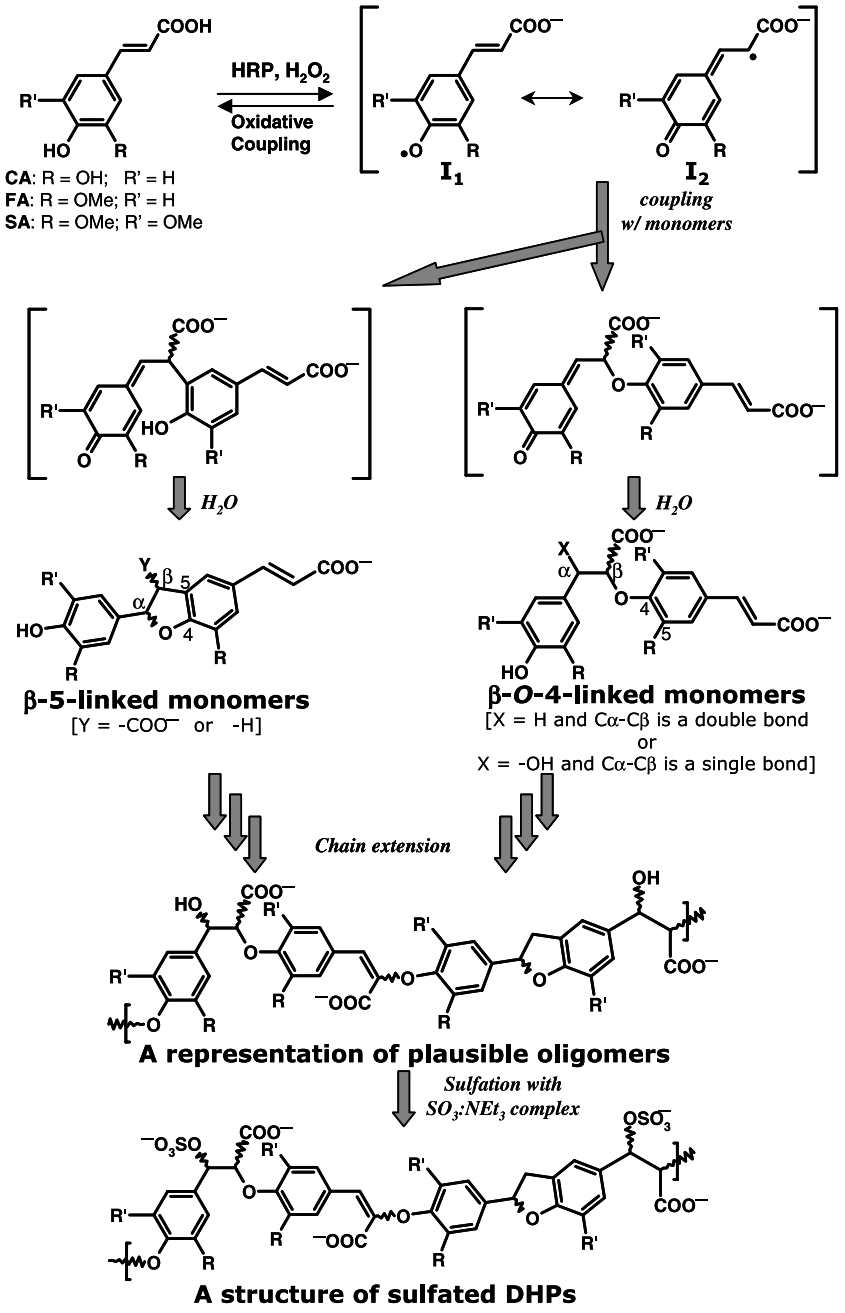

Figure 1. Chemo-enzymatic synthesis of 4-hydroxycinnamic acid-based dehyropolymers (DHPs), CD, FD, and SD. Horseradish peroxidase (HRP)-catalyzed oxidative coupling of caffeic acid (CA), ferulic acid (FA) or sinapic acid (SA) in the presence of H_2O_2 generates oligomers of size 4–15 U, which are sulfated with Et_3N/SO_3 complex to give sulfated DHPs. Phenolic oxidation can generate four types of radical intermediates, of which typically intermediates I_1 and I_2 couple with starting material to give oligomers with different types of inter-monomeric linkages. The most common linkages formed include β-*O*-4 and β-5. Other less common linkages include β-β, 5-5, and 5-*O*-4, for which oligomerization tends to arrest chain growth.[25] The length of the chain greatly depends on the conditions of oligomerization.

and 13-mer for CD, 8 and 15-mer for FD, and 4 and 11-mer for SD.

The DHPs were sulfated using Et_3N/SO_3 complex under conditions established earlier to obtain sulfated oligomers CD_S, FD_S, and SD_S.[14] In this reaction, free phenolic and alcoholic groups are converted into organic sulfate groups resulting in an anionic oligomer. To assess the level of sulfation in these oligomers, elemental composition was determined. The C, H, and O composition of CD was found to be similar to SD, while that of FD, especially in the proportion of carbon, is significantly different (Table 1). Likewise, CD_S and SD_S compositions are similar, and unlike that of FD_S. Calculation of the elemental composition of these oligomers assuming a homogeneous decamer with β-*O*-4 inter-monomer linkage indicates striking similarity to the observed composition for FD and FD_S oligomers. In contrast, the observed composition for the other two DHP derivatives does not match the homogeneous

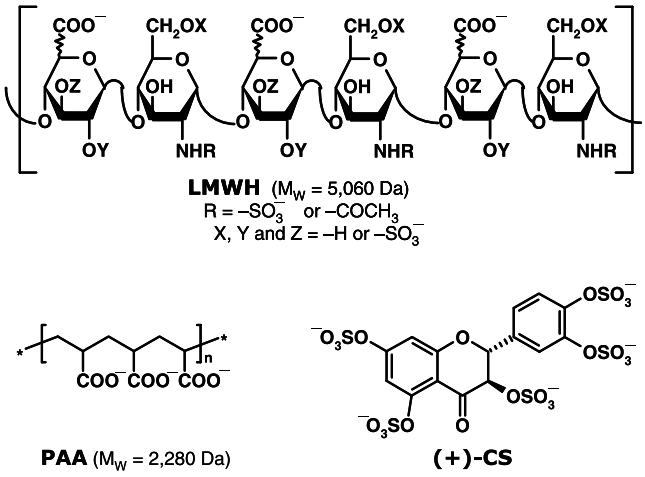

Figure 2. Structures of reference compounds, low molecular weight heparin (LMWH), polyacrylic acid (PAA), and (+)-catechin sulfate ((+)-CS). The average molecular weights (M_W) for polymers LMWH and PAA are as specified, whereas (+)-CS has a molecular weight of 814 Da. LMWH is a heterogeneous mixture of oligosaccharide chains, in which the uronic acid can be iduronic or glucuronic acid, while the glucosamine can be *N*-sulfated or *N*-acetylated (R = $-SO_3^-$ or $-COCH_3$). The 2-, 3-, and 6-positions of saccharide residues can be either sulfated or unsulfated (*X*, *Y*, and *Z* = –H or $-SO_3^-$).

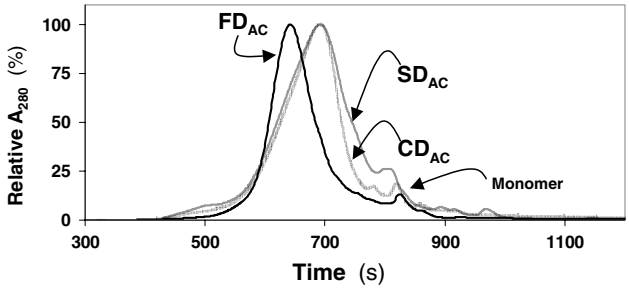

Figure 3. Non-aqueous size-exclusion chromatography of acetylated DHPs. Analytical SEC was performed with ~100–150 μg of CD_{AC} (·····), FD_{AC} (——), and SD_{AC} (——) oligomers using dry THF as the mobile phase at 0.75 mL/min. Each chromatogram is scaled to the highest peak intensity (set to 100%). The time of monomer elution is indicated.

decamer calculation (Table 1). This suggests greater proportion of structurally similar oligomers in FD and FD_S preparations, while CD and SD (also CD_S and SD_S) are expected to be more heterogeneous. The sulfur proportion in the sulfated DHPs remains fairly consistent in the range of 4.4–5.3%, which corresponds to the presence of nearly one sulfate group every 2.5–3.3 monomers. Among the three derivatives, FD_S is the least sulfated preparation (Table 1).

An important question to address with regard to these heterogeneous and polydisperse oligomers was the ease and reproducibility of their synthesis. In multiple attempts on several different scales (up to a gram of the oligomer), the polydispersity of samples as judged by SEC remained consistent suggesting a fairly reproducible process. In addition, the two-step synthesis is a controlled oligomerization process with greater than 60% isolated yield.

2.2. Characterization of dehydrogenation polymers (DHPs)

The IR spectra of sulfated and unsulfated DHPs (Fig. 4) show the presence of aromatic structures (1550 cm^{-1}), as expected. In addition, sulfated DHPs show peaks at 1080 and 1110 cm^{-1} characteristic of sulfate stretches. The ^1H NMR spectra of DHPs show the presence of broad peaks indicating polydispersity (not shown). In contrast, quantitative ^{13}C NMR spectra, recorded with inverse gated decoupling sequence, are more revealing (Fig. 4). Comparison with literature data[26–28] on ^{13}C NMR spectra of dehydro-dimers and -trimers of cinnamic acid derivatives as well as lignin samples suggests that the 165–172 ppm signals belong to carbonyl carbon of carboxylic acid, the 110–150 ppm signals belong to aromatic and vinylic carbons, the 65–100 ppm signals are due to alkoxy carbons C_α and C_β, while the signal between 56 and 60 ppm is because of the $-OCH_3$ group. Yet, significant differences exist between the oligomers. For example, whereas C_α and C_β peaks are prominent in the 65–110 ppm region for SD and CD (Figs. 4A

Table 1. Average molecular weight, elemental composition, and sulfate density of DHPs from cinnamic acid derivatives

DHP	Average molecular weight[a]				Elemental composition[f]				Sulfates per Unit[g]
	M_P[b] (Da)	M_N[c] (Da)	M_W[d] (Da)	Size[e]	C (%)	H (%)	O (%)	S (%)	
CD	1180	880	2800	5–13	54.8 (61.0)[h]	3.5 (3.0)	39.2 (36.0)	—	—
FD	2480	1870	3650	8–15	61.9 (62.8)	4.3 (3.8)	33.6 (33.4)	—	—
SD	1190	1020	2990	4–11	55.8 (59.7)	4.2 (4.2)	38.0 (36.1)	—	—
CD_S	—[i]	—	—	—	46.1 (49.6)	4.0 (2.2)	36.7 (38.1)	5.3 (5.9)	~0.40
FD_S	—	—	—	—	52.4 (54.1)	4.6 (3.1)	35.1 (35.3)	4.4 (4.3)	~0.30
SD_S	—	—	—	—	45.8 (50.4)	4.0 (3.4)	39.0 (37.8)	4.5 (4.9)	~0.38

[a] Average molecular weight was obtained through non-aqueous SEC on the acetylated derivatives, CD_{AC}, FD_{AC}, and SD_{AC}, using polystyrene as standards. The error in determination of these numbers is less than 10% (see Section 4).
[b] Peak-average molecular weight.
[c] Number-average molecular weight.
[d] Weight-average molecular weight.
[e] Size of an average oligomer.
[f] Analysis was performed on unsulfated or sulfated DHPs, and not on acetylated DHPs.
[g] Average number of sulfates per monomeric unit was calculated from elemental sulfur composition and the size of an average unsulfated oligomer.
[h] Numbers in brackets show the predicted composition of a homogeneous β-*O*-4-linked decamer of appropriate DHP.
[i] Not determined.

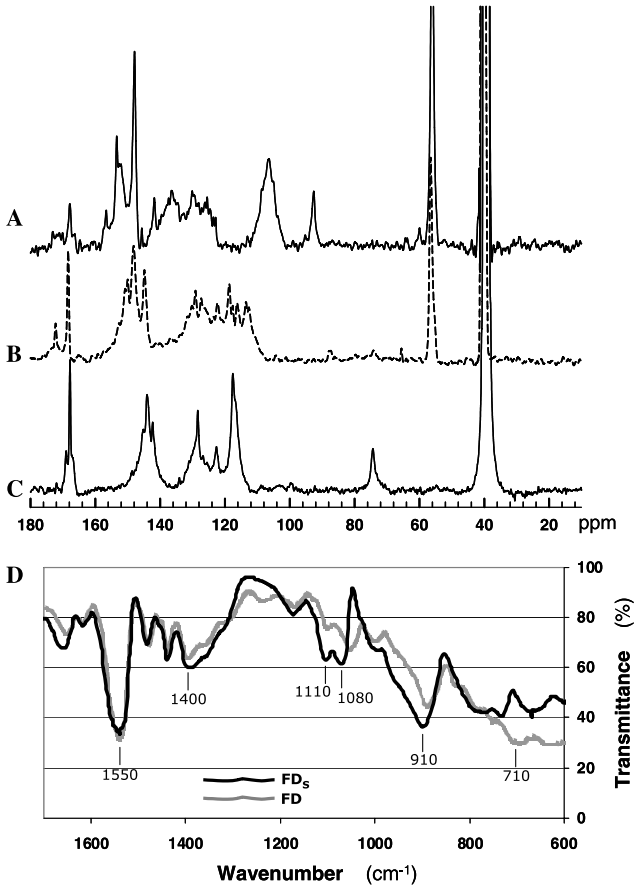

Figure 4. Quantitative ^{13}C NMR spectra of DHPs, SD (A), FD (B), and CD (C) in DMSO-d_6 obtained using inverse gated decoupling pulse sequence. Four regions are apparent, the carboxylic acid region between 160 and 170 ppm, the aromatic and vinylic peaks between 100 and 160 ppm, the α and β-carbons of alkoxy groups between 70 and 100 ppm, and the methoxyl groups at 56 ppm. Solvent signal is observed at ~40 ppm. Note the variation in intensity of carboxylic acid carbon at ~165 ppm and the significant difference in signal composition between the three oligomers. (D) Shows the fingerprint region in the IR spectrum of FD (light line) and FD_S (dark line) in KBr pellets. Sulfate stretches in FD_S are observed at 1080 and 1110 cm^{-1}.

and C), they are nearly absent in this region for FD (Fig. 4B). In addition, the carbonyl carbon intensities for SD and CD are ~25% and 10% lower than that for FD, respectively. Thus, two of the common side-reactions in oxidative coupling—decarboxylation of −COOH groups followed by reaction with a phenoxy radical—seem to have occurred less for FD. Although some decarboxylation is noticeable for CD, the presence of a prominent signal at 68 ppm and the much lower dispersion in the aromatic region indicative of minimal vinylic composition suggest greater possibility of several different types of inter-monomeric linkages (see Fig. 1). In contrast, ^{13}C NMR studies, in combination with elemental analysis, suggest that oligomer FD consists primarily of inter-monomeric linkages of β-*O*-4 type.

Mass spectrometry is an important technique used in elucidation of fine structure of polyphenol polymers, although the heterogeneity and complexity of these molecules presents a formidable challenge.[29,30] Attempts to identify higher oligomeric chains have not yielded much success because of instability of the polyphenolic structure even under mild ionization conditions, such as ESI.[30] We explored the applicability of MS to these 4-hydroxycinnamic acid oligomers to possibly identify the inter-monomer linkages and confirm the spectroscopic results. The ESI-MS spectrum of FD in the negative ion mode is shown in Figure 5A. Mass peaks were observed primarily in the region 150–400 *m*/*z*, yet, smaller peaks were observable between 400 and 700 *m*/*z* (inset in Fig. 5A). Mass peaks at *p*1 (681 *m*/*z*), *p*2 (637 *m*/*z*), and *p*3 (593 *m*/*z*) can be ascribed to singly charged tetramers of FD that have sequentially lost 2, 3, and 4 carboxylic acid moieties, respectively, while those at 533, 489, and 445 *m*/*z* (peaks *p*4, *p*5, and *p*6) most likely arise due to a singly charged trimer that has lost 1 through 3 CO_2, respectively (Fig. 5B). Likewise, peaks *p*7 (385), *p*8 (341) and *p*9 (297) are due to $[M_2]^{-1}$, $[M_2–CO_2]^{-1}$ and $[M_2–2CO_2]^{-1}$ mass fragments, while *p*10 (193) is the singly charged ferulic acid monomer. This analysis supports the result that HRP-catalyzed oxidative coupling of FD gives β-*O*-4-type inter-ferulic acid linkages with the presence of vinylic double bonds in the oligomer (Fig. 5B). Yet, this interpretation does not completely exclude other inter-monomer linkages, which may be present in smaller proportion as can be noted from the large number of as yet uncharacterized peaks in the ESI-MS.

A final point regarding the structure of our DHPs is that current spectroscopic data are insufficient to make definite stereochemical interpretation regarding olefinic linkages as well as substitution at the C_β position (see Fig. 1). The biosynthesis of lignins is largely considered to be achiral[25] and hence, we speculate that multiple isomers are present in the mixture of our oligomers.

2.3. Prolongation of clotting time

Prothrombin and activated partial thromboplastin time (PT and APTT) reflect the activity of the extrinsic and intrinsic pathways of coagulation and thus, are measures of the anticoagulation state of the plasma. PT and APTT were measured with citrated human plasma at six to eight concentrations of unsulfated and sulfated DHPs, while PAA, (+)-CS, and LMWH (Fig. 2) served as reference molecules. All samples showed considerable concentration-dependent prolongation of clotting time (Fig. 6) characterized by a rapid increase in time to clot. The anticoagulant activity is typically defined in terms of the concentration of the anticoagulant needed for doubling the normal plasma clotting time. A 2-fold increase in prothrombin time required plasma concentration of unsulfated DHPs in the range of 98–212 μg/mL (Table 2). This concentration decreased to 42–105 μg/mL, or nearly 2- to 3-fold lower, for sulfated DHPs. In contrast, for the reference molecules PAA and (+)-CS, a massive 4259 and 927 μg/mL concentration was required to achieve doubling of PT, while for a LMWH (from Sigma) 142 μg/mL was sufficient.

In a similar manner, doubling of APTT required ~25–40 μg/mL and ~13–23 μg/mL of unsulfated and sulfated

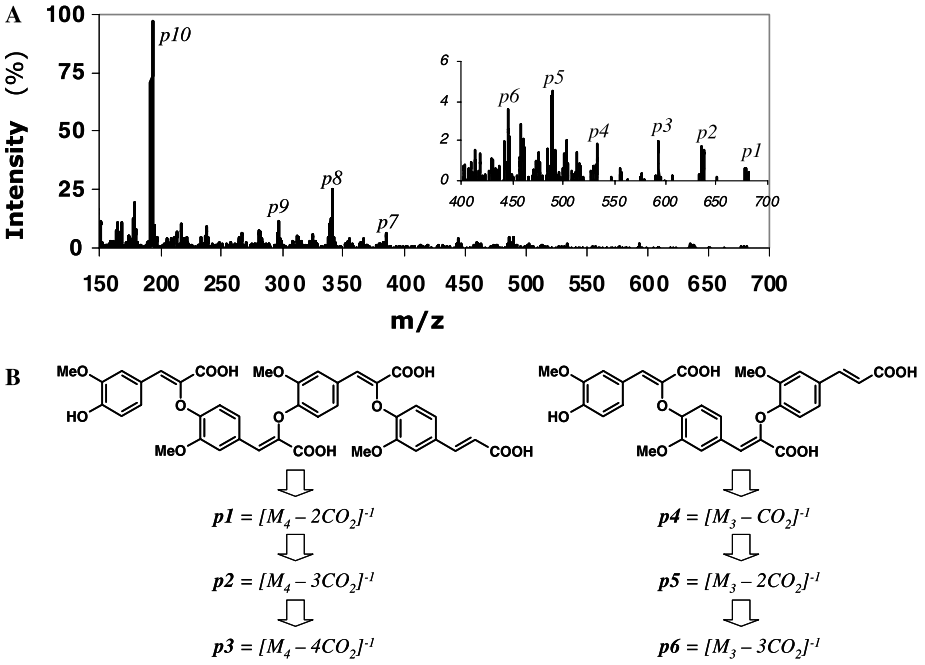

Figure 5. ESI-MS (negative ion mode) of FD oligomer through direct infusion of the oligomer (A) and mass analysis of peaks *p*1 through *p*6 using β-*O*-4 inter-monomeric linkages (B). Peaks merge into the background above 700 amu. A majority of the significant peaks, especially in the higher mass range, can be explained on the basis of a mono-charged molecular ion, which has lost 1 through 3 CO_2 units. See text for details.

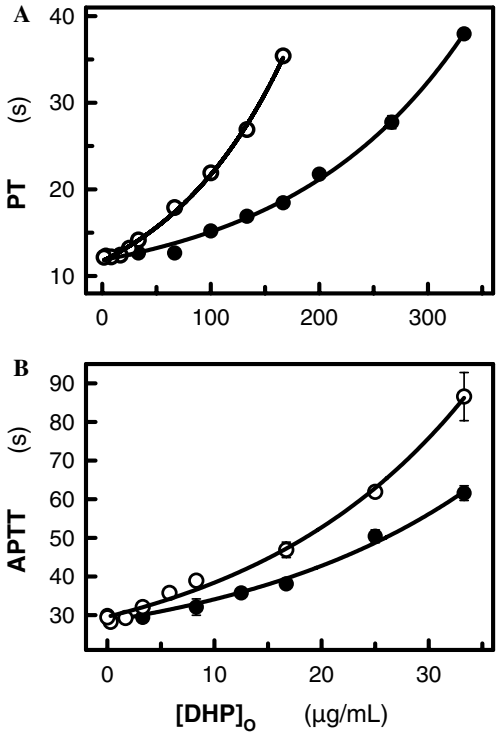

Figure 6. Prolongation of clotting time as a function of SD (closed circles) and SD_S concentrations (open circles) in either prothrombin time assay (A) or the activated partial thromboplastin time assay (B). The solid lines are trend lines, and not exponential fits. Error bars in the range of symbol size have been omitted. In both assay, sulfation of DHP enhanced the anticoagulant activity of oligomer. See Section 4 for details.

DHPs, respectively. In contrast, the LMWH brought about 2× APTT at 5.9 μg/mL. These results suggest that unsulfated DHPs are less potent than their sulfated counterparts. In addition, a trend is discernible. Except for APTT with unsulfated DHPs, CD appears to be consistently more potent than FD, which in turn is better than SD and this trend holds for their sulfated derivatives.

Assuming homogeneous CD, FD, and SD preparations with M_W of 2800, 3650, and 2990 Da, respectively (Table 1), ~35–71 μM and ~15–35 μM of unsulfated and sulfated oligomers, respectively, would be needed for 2× PT. These concentrations change to 9–11 μM and 5–8 μM, respectively, for a doubling of APTT. In comparison, a homogeneous chain of PAA (M_W = 2280 Da) will have to be present at 1870 μM for 2× PT, or at nearly 25- to 125-fold higher level over DHPs. For our small molecule reference, (+)-CS, ~1140 and 350 μM will be needed for doubling of PT and APTT, respectively, which represent ~16- to 76-fold and ~32- to 70-fold higher levels than DHPs. But more importantly, the LMWH (M_W = 5060 Da) gives values of 28 and 1.2 μM for 2× PT and 2× APTT, respectively. Thus, the concentration of DHPs, especially sulfated DHPs, required to double PT is similar to LMWH, while for doubling of APTT ~3- to 10-fold more sulfated DHPs are required.

2.4. Direct and indirect inhibition of coagulation proteinases

To investigate whether our DHPs affect proteolytic activities of coagulation proteinases present in plasma, of which factor Xa and thrombin are probably the most important, we measured residual enzymatic activity of the two enzymes following incubation for a defined time period with varying concentrations of DHPs in the presence and absence of antithrombin. The proteinase

Table 2. Anticoagulation effect of DHPs from 4-hydroxycinnamic acids

DHP	Clotting time[a]		IC_{50}[b]			
	2× PT (μg/mL)	2× APTT (μg/mL)	fXa (μg/mL)	fXa w/AT (μg/mL)	T (μg/mL)	T w/AT (μg/mL)
CD	98.1 ± 0.7	24.9 ± 2.3	0.4 ± 0.1[c]	—[d]	0.19 ± 0.03	—
FD	161.3 ± 2.7	39.5 ± 0.8	2.7 ± 0.3	—	1.16 ± 0.06	—
SD	212.0 ± 2.9	32.1 ± 1.5	2.8 ± 0.9	—	1.00 ± 0.04	—
CD_S	42.1 ± 0.3	13.0 ± 5.0	0.11 ± 0.01	0.19 ± 0.01	0.07 ± 0.01	0.20 ± 0.02
FD_S	63.4 ± 0.1	18.3 ± 1.9	0.32 ± 0.04	0.56 ± 0.02	0.12 ± 0.01	0.31 ± 0.02
SD_S	104.6 ± 3.9	22.6 ± 1.1	0.84 ± 0.08	0.37 ± 0.01	0.33 ± 0.01	0.16 ± 0.01
LMWH	142.1 ± 3.6	5.9 ± 3.0	—	0.037 ± 0.003	—	0.011 ± 0.001
PAA	4259 ± 40	—[d]	No inh.[e]	No inh.	No inh.	No inh.
(+)-CS	927[f]	284[f]	—[d]	—	—	—

[a] PT and APTT values were deduced in in vitro human plasma experiments where the clot initiator is either thromboplastin or ellagic acid, respectively. Experiments were performed in duplicate or triplicate (see Section 4). Errors represent ±1 SE.
[b] IC_{50} values were determined through direct inhibition of thrombin or factor Xa using a chromogenic substrate hydrolysis assay (see Section 4).
[c] SE ± 1.
[d] Not determined.
[e] No inhibition was observed in buffer containing Ca^{2+} at concentrations lower than 4560 μg/mL.
[f] Experiment performed only once.

activity was determined under pseudo-first order conditions in a spectrophotometric assay using chromogenic substrates Spectrozyme fXa and TH for factor Xa and thrombin, respectively. As the concentration of DHP increases the residual proteinase activity decreases in a sigmoidal manner (Fig. 7), which can be fit to a standard dose-dependence equation to derive the IC_{50} values (Table 2).

All unsulfated DHPs studied inhibited both factor Xa and thrombin in the absence of antithrombin with an IC_{50} value in the range of 0.2–2.8 μg/mL or 0.06–0.90 μM (Fig. 7, Table 2). CD was found to be ~5- to 7-fold more effective than FD and SD. The direct inhibitory activity of the DHPs increases significantly on sulfation with IC_{50} values in the range of 0.07–0.84 μg/mL or 20–250 nM. This suggests that sulfation of all three oligomers improves direct inhibition of both factor Xa and thrombin nearly 3- to 4-fold. In contrast, PAA displayed no direct factor Xa or thrombin inhibitory activity in the presence of buffer containing $CaCl_2$ (Table 2).

Indirect inhibition by sulfated DHPs in the presence of antithrombin yields IC_{50} values of 60–140 nM (0.19–0.56 μg/mL) against factor Xa and 50–80 nM (0.16–0.31 μg/mL) against thrombin (Table 2). Although these activities indicate good indirect inhibition potency of the sulfated DHPs, closer inspection of the data indicates some differences and complexities. The IC_{50} value decreases ~2-fold for SD_S in the presence of antithrombin from that in its absence. The decrease in IC_{50} suggests that the indirect and direct inhibition pathway complement each other for SD_S. In contrast, the IC_{50} value increases ~2- to 3-fold for CD_S and FD_S in the presence of antithrombin from that in its absence (Table 2). Thus, for CD_S and FD_S the two pathways appear to either compete with each other or induce side-reactions that prevent additive effects (see Section 3). These mixed inhibition results indicate interesting differences between these three apparently similar oligomers.

An important difference elucidated in these studies is the level of enzyme inhibition induced with each oligomer.

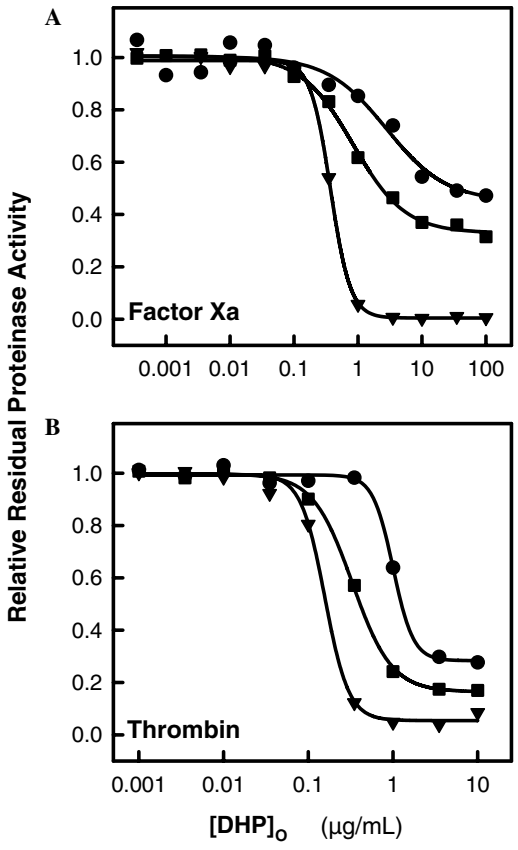

Figure 7. Inhibition of the blood coagulation proteases factor Xa (A) and thrombin (B) by dehydrogenation polymers from sinapic acid: SD (●), SD_S (■), and SD_S in the presence of antithrombin (▼). The inhibition of thrombin and factor Xa by sulfated and unsulfated DHPs was determined through a chromogenic substrate hydrolysis assay. Compared to its unsulfated counterpart, the sulfated DHP was found to be better in inhibiting the procoagulant proteinases. See text for details.

Figure 7 shows that the relative residual proteinase activity decreases to a minimum of ~50%, ~38%, and ~0% for factor Xa, while it reaches ~30%, ~18%, and ~0% for thrombin in the presence of SD, SD_S

and SD_S + AT, respectively. Similar results were also achieved with CD and FD oligomers (not shown). This suggests that even at high enough concentration of unsulfated and sulfated oligomers 100% inhibition of both factor Xa and thrombin was difficult to achieve. In contrast, the inhibition was consistently complete in the presence of antithrombin for all three oligomers. Thus, the presence of antithrombin, or indirect pathway, greatly aided inhibition of both factor Xa and thrombin.

3. Discussion

The template-driven acceleration in antithrombin inhibition of pro-coagulant proteinases, especially thrombin, is extremely attractive because it does not depend on the serpin conformational change phenomenon that is so critically dependent on the structure of the activator.[31] This led to our studies with the carboxylic acid-based scaffold, PAA, which displayed phenomenal acceleration in inhibition, but at physiologically prohibitive concentrations.[15,16] To capitalize on this observation, we sought to introduce two types of functional groups—hydrophobic (−Ar structure) and hydrogen bonds (−OH)—while retaining carboxylic acid (−COOH) groups, so as to enhance the anticoagulant activity.

Macromolecules that satisfy this criteria are difficult to find, except perhaps for synthetic lignins, the so-called DHPs.[20,25,32] However, natural lignins do not contain carboxylic acid moieties. Synthetic lignins containing carboxylic acid groups have been obtained, yet molecules longer than trimers have not been synthesized to date.[23] We utilized HRP-catalyzed oxidative coupling conditions, such as high reactant concentrations and controlled gradual addition of the oxidant, which reduce chain termination, to obtain oligomers with average molecular weight between 800 and 3500 Da. This corresponds to a size range of tetramers to pentadecamers. This is the first time that higher order oligomers of −COOH containing DHPs have been synthesized.

Detailed characterization of all possible inter-monomeric linkages in these DHPs is difficult, yet the ESI-MS spectrum of FD indicates the presence of at least one type of linkage, the β-*O*-4-type (Fig. 5). This is likely to be the major linkage in FD with other linkages, such as β-5, β-β, and 5-5, also present to some extent. In addition, it is likely that not all carboxylic acid groups are retained during oligomerization, as suggested by the ^{13}C NMR spectrum (Fig. 4), due to the competing decarboxylation reaction. Finally, a number of chiral centers are being generated in oxidative β-5 and β-β coupling, which are likely formed without any stereochemical control.[33] Thus, despite the apparent simplicity of ESI-MS spectrum (Fig. 5) the composite structure of FD, and of CD and SD too, is likely to be complex. This structural complexity is expected to be retained in sulfated DHPs.

The presence of so many structures in a single preparation of DHPs is both a blessing and a curse. While heterogeneity and polydispersity imply that reliable, discrete structural information on molecules that possess activity is difficult, they also afford an essentially high-throughput screening of a large library, which in this case is combinatorial because of the nearly random coupling of radicals (see Fig. 1). For this work in which the objective was on arriving at effective structure(s), rather than coming up with detailed structural sequence of a potent molecule, screening such a library was especially advantageous. This library represents a group of structures—the 'lignin carboxylates'—because each molecule herein contains the fundamental lignin structure, an aromatic ring, a three-carbon unit, and some −OH groups, with a high probability of possessing carboxylic acid groups.

The anticoagulant properties, assessed under in vitro conditions using PT and APTT assays, suggest that all DHPs studied, sulfated and unsulfated, were fairly potent. In addition, the potency of these DHPs was dramatically greater than our reference polymer, PAA (Table 2), suggesting that our reasoning based on first principles—hydrophobicity and anionic character—appears to have succeeded. More importantly, the results indicate that unsulfated CD, FD, and SD were similar in potency to a LMWH in PT assays. This result is striking considering that LMWHs are currently being used in the clinic. More experiments are needed to better understand the anticoagulant efficacy of our DHPs.

In contrast to unsulfated DHPs, their sulfated counterparts were nearly 1.4- to 3.4-fold more effective than LMWH in PT assays (Table 2). In APTT assays, the unsulfated DHPs were 4.2- to 6.7-fold weaker than LMWH, while the sulfated DHPs were 2.2- to 3.8-fold less effective. Thus, sulfation enhanced the anticoagulation potency of DHPs. Overall, both sulfated and unsulfated DHPs are more effective at prolonging the APTT than the PT (Fig. 6, Table 2). LMWH also behaves in a similar fashion. This suggests that DHPs affect the intrinsic pathway of coagulation more than the extrinsic pathway, similar to LMWHs.

Among the six DHPs studied, the anticoagulant potency followed an order $CD_S > FD_S > SD_S \cong CD$. Although it is difficult to derive precise structural information, it is tempting to suggest that the order of potency possibly follows the number of anionic group density in these oligomers. Yet, calculations using the sulfate content (Table 1) and carbonyl carbon intensities (Fig. 4) reveal that the negative charge ($-COO^-$ and $-OSO_3^-$) density follows the order $CD_S = FD_S > SD_S > CD$. Further, although the anticoagulant activity of CD_S is similar to that of LMWH, its charge density is significantly lower.[9–11,34] Thus, while the negative groups are important for enhancing activity, they are by no means the only determinant of anticoagulant activity.

With respect to structural diversity of these preparations, SD is the least diverse because of the presence of two methoxy groups in the aromatic ring, which reduce the formation of 5-5 and β-5 linkages. In contrast, CD with one hydroxy group, which encourages such

linkages, is the most diverse.[25] Thus, it is likely that an optimal combination of structural diversity and anionic character generates the anticoagulant property in these DHPs.

Mechanistic differences between DHPs and LMWH are revealed in experiments on direct and indirect inhibition of thrombin and factor Xa (Fig. 7, Table 2). All DHPs, sulfated and unsulfated, inhibited the hydrolysis of an appropriate chromogenic substrate by both factor Xa and thrombin directly in the absence of antithrombin! This is quite an un-expected result considering that PAA does not display this behavior. It is likely that the significant hydrophobic character of these DHPs is the origin for this direct inhibition effect.

The IC_{50} values of these effects were ~4- to 135-fold below the lowest concentration necessary for a 2-fold increase in APTT suggesting that direct inhibition of these proteinases is most probably a relevant mechanism of action of these anticoagulants. As observed in clotting time assays, sulfated DHPs were more effective at inhibiting both proteinases than unsulfated counterpart by a factor of ~2.6- to 9.7-fold. Likewise, CD and CD_S were better anticoagulants than the other two DHPs in these assays and probably for the same reasons of structural diversity and charge density.

Inhibition of thrombin and factor Xa with DHPs in the presence of antithrombin exhibited greater complexity. While the IC_{50} value against thrombin and factor Xa improved nearly 2-fold for SD_S, it worsened 2- to 3-fold for CD_S and FD_S. Indirect inhibition of thrombin and factor Xa with these anticoagulants is a complex process because of two simultaneous processes—direct and basal inhibition. Thus, indirect inhibition is an additive function of the two processes. A pathway that competes with the indirect effect and reduces its efficiency is the substrate pathway.[35,36] This substrate pathway regenerates a fully active enzyme from the covalent antithrombin–enzyme complex, thereby raising the stoichiometry of inhibition.[35] It is possible that some molecules present in heterogeneous CD_S and FD_S preparations introduce the substrate pathway component, thereby decreasing the overall efficacy of inhibition in the presence of antithrombin.

At a molar level, comparison of the IC_{50} value in the presence of antithrombin (indirect inhibition) suggests that DHPs are 8- to 20-fold weaker factor Xa inhibitors and 24- to 38-fold weaker thrombin inhibitors than LMWH (Table 2). The proportions do not change much for direct inhibition of these two enzymes (~5- to 35-fold against fXa and 10- to 48-fold against thrombin). This observation suggests that although DHPs can utilize the antithrombin-dependent (indirect) pathway, the antithrombin-independent (direct) pathway is the major contributor to overall proteinase inhibition.

With the limited knowledge we have on these novel oligomers at the present time, it remains to be determined whether this represents a significant divergence from the effect of traditional anticoagulant, LMWH, which is known to predominantly utilize the indirect pathway.[31,34] Recent studies suggest that LMWH may also function through inhibition of intrinsic tenase complex, which is independent of antithrombin.[37] Likewise, heparin is known to interact with an exosite on factor IXa, which plays an important role in antithrombin-independent inhibition of intrinsic tenase.[38,39]

Although the heterogeneous and polydisperse nature of these DHP oligomers suggests an intrinsic difficulty of deriving useful structural information for rational design of advanced molecules, the results present a wealth of opportunities. The molecules present in the mixture have a common structure, the 'lignin carboxylate'. Assuming that a decamer is the minimal size necessary for anticoagulant function, a combinatorial virtual library can be prepared with the type of monomer, for example, CA, FA, and SA (Fig. 1), and inter-monomer linkages, for example, β-*O*-4, β-5, β-β, and 5-5, as variables. A simple calculation shows that 262,144 different decamer structures are possible for each monomer from these four inter-monomer linkages. This library size is well within the reach of virtual screening techniques. Thus, our current 'structure(s)' search is expected to generate some novel first generation structures for traditional organic synthesis and biochemical evaluation.

In conclusion, preliminary results suggest that our chemo-enzymatically prepared structurally complex DHPs from 4-hydroxycinnamic acids display interesting anticoagulant activities with the potency of CD_S resembling LMW heparin in in vitro plasma clotting time assays. In addition, the anticoagulant function appears to originate from both antithrombin-dependent and -independent mechanisms with the indirect pathway appearing to be the major contributor. This dual inhibition property of our DHP oligomers is the first example in inhibitors of coagulation. Yet, detailed mechanistic studies are necessary to better understand the mode of action of these novel oligomers. These molecules are structurally unlike the highly sulfated polysaccharide scaffold of heparins and are readily obtained in few steps. This work puts forward novel, non-heparin structure(s), which may be exploited for the design of potent, dual action inhibitors of coagulation through combinatorial virtual screening on a library of DHP oligomers.

4. Experimental

4.1. Proteins and chemicals

Horseradish peroxidase (HRP) with activity of 250–330 U/mg was from Sigma (St. Louis, MO). Human antithrombin (AT) was from molecular innovations (Southfield, MI), and proteinases, factor Xa, and thrombin were from Haematologic Technologies (Essex Junction, VT). Stock solutions of proteins were prepared in 20 mM sodium phosphate buffer, pH 7.4, containing 100 mM NaCl (AT and thrombin) or 5 mM MES buffer, pH 6.0 (factor Xa). Chromogenic substrates Spectrozyme TH and Spectrozyme fXa were from American Diagnostica (Greenwich, CT). Citrated human plasma

for coagulation time assays was purchased from Valley Biomedical (Winchester, VA). Thromboplastin and ellagic acid were obtained from Fisher Diagnostics (Middletown, VA). LMWH (M_W = 5060 Da), 30% hydrogen peroxide, sinapic acid (SA), ferulic acid (FA), and caffeic acid (CA) were from Sigma (St. Louis, MO). PAA2280 was from American Polymer Standards (Mentor, OH), while (+)-CS (Fig. 2) was prepared as described earlier.[12–14] All other chemicals were of analytical reagent grade from either Aldrich Chemicals (Milwaukee, WI) or Fisher (Pittsburgh, PA) and used without further purification.

4.2. Oxidative coupling and sulfation of 4-hydroxycinnamic acid derivatives

The dehydrogenation polymers (DHP) were prepared by the so-called 'zutropfverfahren' procedure consisting of slow addition of a phenylpropenoid precursor and H_2O_2 to a solution of horseradish peroxidase (HRP).[21,24] Briefly, 4-hydroxycinnamic acid precursor (25 mM) in 10 mM sodium phosphate buffer, pH 8.0, (200 mL) and H_2O_2 (75 mM) in the same buffer (100 mL) were simultaneously added drop-wise over a 5 h period to a stirring solution of HRP (10 mg) in the sodium phosphate buffer (50 mL) at room temperature in dark. Three additional aliquots of H_2O_2 (75 mM) were added over the next 72 h while monitoring the polymerization using analytical size-exclusion chromatography (SEC). At the end of nearly 80 h, the solution was freeze-dried, the solid re-dissolved in deionized water, and the solution filtered repeatedly through a molecular membrane (Amicon YM5K) to remove salts and low molecular weight material. The final solution was washed with ether and lyophilized to give a dark brown powder, the sodium salt of the DHP. The synthetic DHPs were sulfated with triethylamine–sulfur trioxide complex.[14,40] Briefly, the lyophilized DHP sample (500 mg) was dissolved in dry DMF (50 mL) containing triethylamine–sulfur trioxide complex (1 g) and stirred for 24 h at 60 °C. After the removal of most of the DMF in vacuo, the remaining product was taken up in 30% aqueous sodium acetate, the sodium salt precipitated using ~10 volume of cold ethanol. The precipitated product was further purified with dialysis using Amicon 10K cutoff dialysis membrane.

4.3. Characterization of DHPs

Elemental analysis of DHP samples was obtained from Atlantic Microlabs (Norcross, GA). Infrared spectra were recorded on a Thermo Nicolet Avatar 360 FT-IR spectrophotometer (Somerset, NJ) with DHP samples in KBr pellets. The molecular weights of acetylated DHPs were determined using a Phenogel 500 Å non-aqueous size-exclusion column (300 × 7.8 mm, Phenomenex, Torrance, CA). Acetylation of DHPs was performed using a standard Ac_2O/pyridine (1:1) mixture for 24 h at room temperature. Acetylated DHPs were eluted with THF at 0.75 mL/min[41] and detected at 280 nm. Polystyrene samples (450–7600 Da) from American Polymer Standards, Mentor, OH, were used as standards. Number-average molecular weights (M_N) and weight-average molecular weights (M_W) were calculated by dividing the base of each peak into 10 equal intervals. The peak heights at each of these points were determined using CLASS VP (Shimadzu) software and the molecular weights at these points obtained from a standard curve. The M_N and M_W values were then calculated using standard equations. For ^1H and ^{13}C NMR spectroscopy, 5–20% (w/v) solutions of dry DHPs in DMSO-d_6 were prepared. The spectra were recorded on a Gemini 300 spectrometer (Varian, Palo Alto, CA) at 298 K (^1H) or 323 K (^{13}C). Quantitative analysis of ^{13}C signal intensities was performed using inverse gated decoupling sequence with a 10 s relaxation delay and an acquisition time of 1.7 s. Nearly, 25,000–40,000 scans were acquired for signal integration. Mass spectrometry was performed on sulfated and unsulfated DHP samples using a Micromass ZMD4000 single quadrupole mass spectrometer with ESI ionization probe operating in negative ion mode (Waters Corp., Milford, MA). The samples, dissolved in acetonitrile containing formic acid (5% v/v), were infused at 10 μL/min and optimized MS ionization conditions were employed. The source block temperature and the probe temperature were held at 100 and 120 °C, respectively. Corona and cone voltages of 2.69 kV and 161 V were selected following optimization. The desolvation nitrogen flow was 500 L/h. Mass spectra were acquired in the mass range from 110 to 1000 Da at 400 amu/s.

4.4. Prothrombin time and activated partial thromboplastin time

Clotting time was determined in a standard 1-stage recalcification assay with a BBL Fibrosystem fibrometer (Becton–Dickinson, Sparles, MD). For PT assays, thromboplastin was reconstituted according to manufacturer's directions and warmed to 37 °C. A 10 μL sample of the DHP, to give the desired concentration, was brought up to 100 μL with citrated human plasma, incubated for 30 s at 37 °C followed by addition of 200 μL pre-warmed thromboplastin. Clotting time in the absence of an anticoagulant was determined using 10 μL deionized water. For APTT assay, 10 μL DHP sample was mixed with 90 μL citrated human plasma and 100 μL of pre-warmed APTT reagent (0.2% ellagic acid). After incubation for 220 s, clotting was initiated by adding 100 μL of 25 mM $CaCl_2$ (37 °C) and time to clot noted. Each clotting assay was performed in duplicate or triplicate. The data were fit to a quadratic function, which was used to determine the concentration of DHP (or the reference molecules) necessary to double the clotting time, 2× APTT or 2× PT.

4.5. Proteinase inhibition

Both direct and indirect inhibition of thrombin and factor Xa by sulfated and unsulfated DHPs was determined through a chromogenic substrate hydrolysis assay. A 10 μL sample of DHP at concentrations ranging from 0.035 to 10000 μg/mL was diluted with 885 μL of 20 mM Tris–HCl buffer, pH 7.4, containing 100 mM NaCl, 2.5 mM $CaCl_2$, and 0.1% PEG8000 at room temperature in polyethylene glycol-coated polystyrene

cuvettes, followed by addition of 5 μL of proteinase solution to give 4 nM thrombin or factor Xa. After 10 min of incubation at room temperature, 100 μL of 1 mM chromogenic substrate was added (Spectrozyme fXa for factor Xa and Spectrozyme TH for thrombin) and the residual proteinase activity was determined from the initial rate of increase in absorbance at 405 nm. Relative residual proteinase activity at each concentration was calculated using the proteinase activity measured under otherwise identical conditions, except for the absence of DHP. Indirect inhibition of thrombin and factor Xa using antithrombin–sulfated DHP complex was performed in the presence of 100 and 200 nM antithrombin for inhibition, respectively, in an otherwise identical manner. The sigmoidal dose-dependence of residual proteinase activity was fitted with a logistic function of the form $f(x) = Y_o + (1 - Y_o)/(1+([DHP]_o/IC_{50})^{HS})$, where Y_o is the lower threshold level of the inhibitory activity and HS is the Hill-slope.

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