Effects of Tannins from *Geum japonicum* on the Catalytic Activity of Thrombin and Factor Xa of Blood Coagulation Cascade

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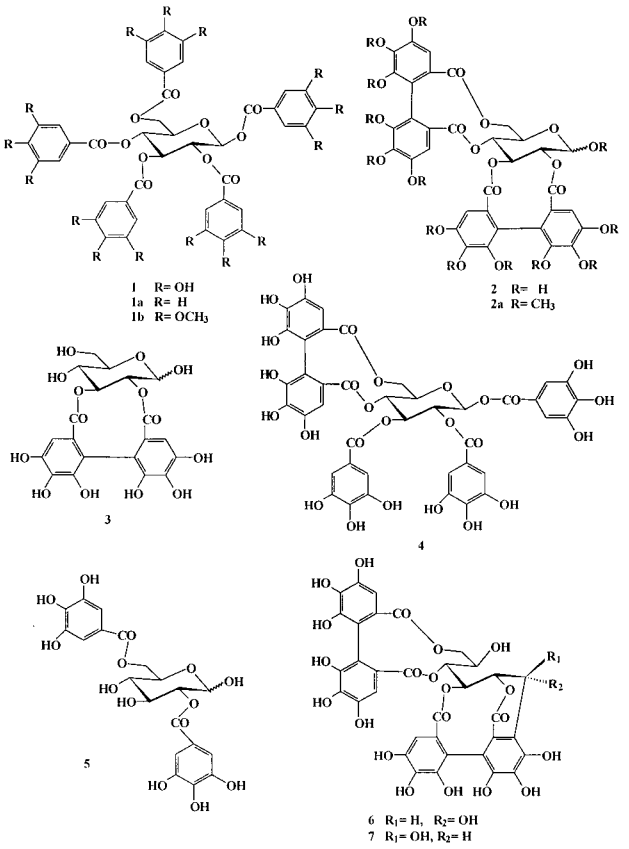
Bioassay-guided fractionation of the MeOH extract of the whole plant of *Geum japonicum* led to the isolation of seven known tannins. They were identified by spectroscopic methods as penta-*O*-galloyl-*β*-glucoside (**1**), pedunculagin (**2**), 2,3-(*S*)-hexahydroxydiphenoyl-D-glucose (**3**), tellimagrandin II (**4**), 2,6-di-*O*-galloyl-D-glucose (**5**), casuariin (**6**), and 5-desgalloylstachyurin (**7**). Compounds **1**, **2**, **4**, **6**, and **7** showed potent anticoagulant activity by significantly prolonging the clotting of rabbit plasma. The inhibitory effect of **2** was competitively directed against thrombin. Its IC_{50} values for inhibition of the enzymatic activity of thrombin on synthetic substrate and fibrinogen were 0.18 and 0.15 μM, respectively. On the other hand, compounds **1**, **4**, **6**, and **7** are mixed noncompetitive inhibitors of thrombin. Their IC_{50} values for inhibition of fibrinogen hydrolysis were twofold to sevenfold lower than those for the inhibition of synthetic substrate hydrolysis. Factor Xa was competitively inhibited by compounds **1**, **2**, **4**, **6**, and **7**. The phenolic hydroxyl groups of the active tannins appear to play an important role in their inhibitory effect on the enzymes.

Blood coagulation is a complex process involving initiation, the formation of the fibrin clot and platelet plug, and localization of the clot to the area of vascular injury.



Thrombin plays a central role in the blood coagulation cascade by catalyzing the conversion of fibrinogen to fibrin, leading to fibrin thrombus formation. A safe and effective inhibitor of thrombin could be a useful tool in the treatment of venous thrombosis, arterial fibrillation, restenosis, and arterial thrombosis and in the prevention of myocardial infarction. Because of this, the modulation of thrombin by direct, small molecule inhibitors is a widely sought goal in the development of anticoagulant agents.[1] The regulation of thrombosis by using thrombin inhibitors has been studied extensively. All these inhibitors reported so far are either peptides or proteins from both natural and synthetics sources.[2,3]

The whole plant of *Geum japonicum* Thunb. (Rosaceae) has been used as a diuretic and an astringent in China and Japan.[4] Several tannins and anti-HIV triterpenes have been isolated from this plant.[4−6] In the course of screening for anticoagulant agents from plants, we found that the methanol extract of the whole plant of *G. japonicum* exhibited potent anticoagulant activity. In this paper, we report the isolation of seven tannins from *G. japonicum* and evaluate their anticoagulant effects, particularly on thrombin and factor Xa activities.

Results and Discussion

Seven known tannins, penta-*O*-galloyl-*β*-D-glucose (**1**),[7] pedunculagin (**2**),[8] 2,3-(*S*)-hexahydroxydiphenoyl-D-glucose (**3**),[9] tellimagrandin II (**4**),[8] 2,6-di-*O*-galloyl-D-glucose (**5**),[10] casuariin (**6**),[8,11] and 5-desgalloylstachyurin (**7**)[12] were isolated from the methanol extract of the whole plant. Their structures were determined by spectroscopic methods and by comparison with data reported in the literature.

The anticoagulant activity of these seven tannins was determined by measuring their ability to prolong clotting of rabbit plasma. Compound **1** significantly prolonged the clotting times of rabbit plasma over 300 s in the prothrombin time (PT), the thrombin time (TT), and the activated partial thromboplastin time (APTT) assays with concentrations of 0.45, 0.50, and 0.47 mM, respectively (Figure 1). Compounds **2**, **4**, **6**, and **7** also showed significant anticoagulant activity with clotting times greater than 300 s in

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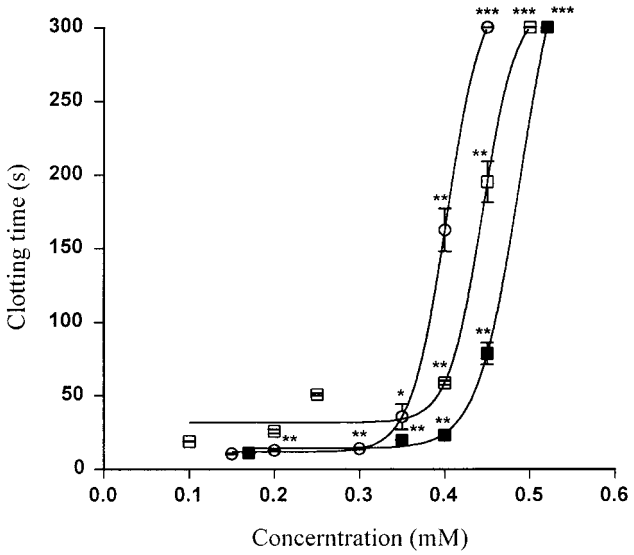

Figure 1. Effect of **1** on prothrombin time (PT, ○), thrombin time (TT, ■), and activated partial thromboplastin time (APTT, □) clotting assays. Each point is reported as mean (seconds) ± SD ($n = 3-5$); * $p < 0.05$, ** $p < 0.01$, significantly different from control, Student's *t*-test. *** clotting time > 300 s.

the PT, TT, and APTT assays at a concentration of 1 mM. On the other hand, compounds **3** and **5** had no effect on plasma clotting at concentrations up to 2 mM (Table 1). These results indicated that compounds **1**, **2**, **4**, **6**, and **7** inhibited either both intrinsic and extrinsic pathways or the common pathway in the blood coagulation cascade. The positive control, heparin, showed significant anticoagulant effects in APTT and TT assays rather than in the PT assay, with a lower concentration (1 (g/mL) (Table 1). These results are in accordance with those reported previously.[13]

As a first step in characterizing the anticoagulant effects of these tannins, their effect on proteolytic activity of thrombin on synthetic peptide substrate was studied. The IC_{50} values of **1**−**7** are presented in Table 2. Compounds **1**, **2**, **4**, **6**, and **7** exhibited a dose-dependent inhibition of thrombin-catalyzed hydrolysis of the chromogenic substrate, H-D-Phe-Pip-Arg-*p*-nitroanilide (S-2238). Among them, compound **4** showed the most potent activity, with an IC_{50} of 0.070 μM. Compounds **6** and **7** are anomers with a different configuration at the C-1 position of the sugar moiety. The inhibitory activity of compound **7** on thrombin is about twofold more potent than that of compound **6** (Student's *t*-test, $p < 0.05$). Under the same experimental conditions, compounds **3** and **5** exhibited no measurable inhibition of thrombin-catalyzed reaction at a higher concentration of 200 μM.

The kinetic studies of compounds **1**, **2**, **4**, **6**, and **7** were conducted to determine the type of inhibition and the inhibition constants (K_i). Compound **2** competitively inhibited S-2238 hydrolysis by bovine thrombin, with a K_i value of 0.042 μM. On the other hand, compounds **1**, **4**, **6**, and **7** showed mixed noncompetitive inhibition of S-2238 hydrolysis by bovine thrombin, with K_i values of 3.9, 0.42, 0.98, and 0.83 μM, respectively. These results indicate that compound **2** binds to the active site of thrombin, whereas

Table 1. Effects of **1**−**7**, and Heparin on Prothrombin Time (PT), Thrombin Time (TT), and Activated Partial Thromboplastin Time (APTT) Assays *In Vitro*[a]

compounds (mM)	PT (s)	TT (s)	APTT (s)
control	10.2 ± 0.1	11.2 (0.3	42.6
[b]**1** (0.5)	>300[c]	>300	>300
(0.25)	13.9 ± 0.8**	16.5	50.6 ± 0.9
2 (1.0)	>300	>300	>300
(0.75)	15.2 ± 1.4**	33.5 ± 2.4**	60.2 ± 12.0
(0.50)	13.5 ± 0.2**	22.7 ± 1.6**	43.7 ± 2.3
(0.25)	10.2 ± 1.2	12.6 ± 1.5	35.3 ± 6.5
3 (2.0)	10.0 ± 0.2	9.43 ± 0.1	39.9 ± 4.7
4 (1.0)	>300	>300	>300
(0.50)	11.9 ± 0.1**	18.7 ± 0.6**	45.7 ± 4.1
5 (2.0)	11.1 ± 0.2	10.7 ± 0.1	40.8 ± 3.2
6 (1.0)	>300	>300	>300
(0.5)	13.6 ± 0.3**	12.3 ± 0.3*	72.3 ± 8.0*
7 (1.0)	>300	>300	>300
(0.5)	13.9 ± 0.9**	16.2 ± 0.9**	31.6 ± 2.1
[d]heparin, 500 μg/mL	33.6 ± 0.5**	>300	>300
250 μg/mL	23.7 ± 0.2**	>300	>300
1 μg/mL	10.1 ± 0.3	60.4 ± 6.7**	63.2 ± 6.3*

[a] PT, TT, and APTT were determined in the presence of the indicated concentrations of **1**−**7**. Data are reported as clotting times (seconds) of plasma (mean ± SD, $n = 3-5$); * $p < 0.05$, ** $p < 0.01$ significantly different from control, Student's *t*-test. [b] Other data shown in Figure 1. [c] No clotting was observed (clotting time >300 s). [d] Heparin sodium salt from porcine intestinal, 187 USP units/mg.

Table 2. Inhibitory Activities of Compounds **1**−**7** on Thrombin-Catalyzed Hydrolysis of S-2238 and Fibrinogen[a]

compounds	IC_{50} (μM) for S-2238	IC_{50} (μM) for fibrinogen	type of inhibition	K_i (μM)
1	0.11 ± 0.02[b]	0.050 ± 0.006*	MNC[c]	3.9 ± 0.6
2	0.18 ± 0.01	0.15 ± 0.03	CC[d]	0.042 ± 0.004
3	>200	>200	N. D.[e]	N. D.[e]
4	0.070 ± 0.01	0.014 ± 0.001**	MNC[c]	0.42 ± 0.06
5	>200	>200	N. D.[e]	N. D.[e]
6	0.28 ± 0.02	0.059 ± 0.002*	MNC[c]	0.98 ± 0.2
7	0.19 ± 0.02	0.026 ± 0.002**	MNC[c]	0.83 ± 0.2

[a] IC_{50} values were the concentration inhibiting 50% of the initial activities of enzymes; K_i values were determined as reported under Experimental Section. [b] The data presented are those obtained from the analysis with best fitting; shown as mean ± SD ($n = 3$); * $p < 0.05$, ** $p < 0.01$ significantly different from their IC_{50} values for S-2238 substrate, Student's *t*-test. [c] MNC: mixed noncompetitive. [d] CC: classical competitive. [e] Data not detected.

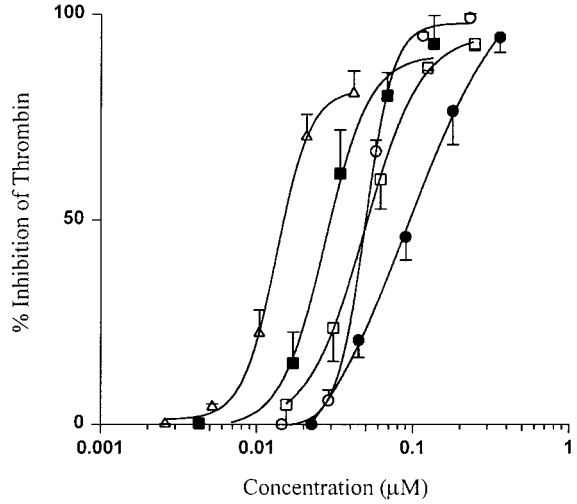

Figure 2. Effect of **1** (○), **2** (●), **4** (△), **6** (□), and **7** (■) on the inhibitory activity of thrombin-induced fibrin-clotting. Points shown are means of triplicate ± standard derivation.

compounds **1**, **4**, **6**, and **7** bind to two different sites, at both the active site of thrombin and a secondary site.

It is known that thrombin in the blood coagulation cascade catalyzes the conversion of fibrinogen to fibrin clots. Therefore, the effect of these tannins on the proteolytic activity of thrombin on its macromolecular substrate, fibrinogen, was examined. Compounds **1**, **2**, **4**, **6**, and **7** exhibited a dose-dependent inhibition of thrombin-catalyzed conversion of fibrinogen to fibrin clots (Table 2, Figure 2). Compounds **1**, **4**, **6**, and **7**, which exhibited mixed noncompetitive inhibition, showed a decrease (twofold to sevenfold) in their IC_{50} values of inhibition of fibrin clot formation (Table 2) compared to those of inhibition of S-2238 hydrolysis. This indicates that the second binding site is most probably the anion binding exosite (ABE)[14] of thrombin. On the other hand, the IC_{50} values of compound **2** for hydrolysis of synthetic and macromolecular substrates were not significantly different (Student's *t*-test, $p > 0.05$). This is expected, as compound **2** is a competitive inhibitor of thrombin and only binds to the active site of thrombin. Among these active compounds, **4** showed the most potent inhibitory activity on the thrombin-induced fibrin clotting, with an IC_{50} of 0.014 μM.

To test the specificity of these tannins, their effects on proteolytic activity of factor Xa were determined. Compounds **1**, **2**, **4**, **6**, and **7** inhibited factor Xa-catalyzed hydrolysis of synthetic substrate S-2222 (*N*-benzoyl-L-isoleucyl-L-arginine-*p*-nitroaniline hydrochloride and its methyl ester). Their IC_{50} values were 0.17, 0.56, 0.21, 0.72, and 0.28 μM, respectively. These values are 1.5 to 3 times less than those for the inhibition of thrombin, indicating only a small preference for thrombin over factor Xa. The kinetics of inhibition of factor Xa for these tannins was also examined. Compounds **1**, **2**, **4**, **6**, and **7** are competitive inhibitors of factor Xa, indicating that they bind only to the active site of the enzyme. Compounds **1**, **4**, **6**, and **7**, which apparently bound to ABE and the active site of thrombin (see above), could bind only to the active site of factor Xa, inasmuch as they are similar in nature. Compound **2**, however, binds to the active site of both enzymes. Thus, these results can be explained by the fact that factor Xa, unlike thrombin, does not have ABE (Figure 3). The K_i value of compound **2** for inhibition of factor Xa was 0.99 μM, and about 23-fold more than that of thrombin. In contrast, compounds **1**, **6**, and **7** showed that the K_i values for inhibition of thrombin are about 1.2−6.8 times that of

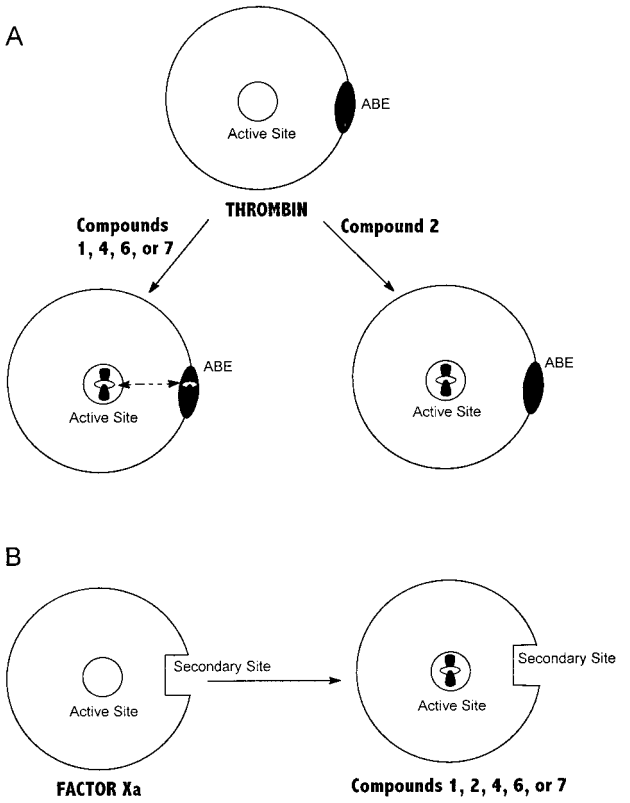

Figure 3. Schematic representation of interaction of tannins with thrombin (A) and factor Xa (B). Compound **2** binds only to the active site of thrombin, whereas compounds **1**, **4**, **6**, and **7** bind to both the active site and ABE (A). On the other hand, all the compounds bind only to the active site of factor Xa (B).

Table 3. Inhibitory Activities of Compounds **1**, **2**, **4**, **6**, and **7** on Factor Xa-Catalyzed Hydrolysis of S-2222[a]

compounds	IC_{50} (μM)	type of inhibition	K_i (μM)
1	0.17 ± 0.08	CC[b]	0.57 ± 0.08
2	0.56 ± 0.05	CC[b]	0.99 ± 0.04
4	0.21 ± 0.02	CC[b]	0.44 ± 0.1
6	0.72 ± 0.3	CC[b]	0.45 ± 0.05
7	0.28 ± 0.04	CC[b]	0.69 ± 0.04

[a] K_i values were determined as reported under Experimental Section. The data presented are shown as mean ± SD ($n = 3$). [b] CC: classical competitive.

factor Xa (Tables 2, 3). Compound **4** competitively inhibited factor Xa, with similar K_i values for inhibition of thrombin and factor Xa.

The formation of tannin-enzyme complexes are usually mediated by hydrogen bonding between phenolic hydroxyl groups and polar groups in the active site of enzyme.[15] To determine the role of the hydroxyl groups of phenolic moieties, compound **1a**, with no phenolic hydroxyl groups, as well as the methylated derivatives, **1b** and **2a**, were synthesized. These three compounds showed no inhibition of thrombin at a concentration up to 100 μM. These results indicate that the phenolic hydroxyl groups of the tannins play a key role in their inhibitory effect on thrombin.

Tannins are well known for their physiological and pharmacological actions.[16] Here, the ability of tannins from *G. japonicum* to inhibit two key serine proteinases of the blood coagulation cascade, thrombin and factor Xa, is demonstrated. The seven tannins studied here can be classified into four types, namely gallotannins (**1** and **5**), ellagitannins (**2** and **3**), C-glycoside ellagitannins (**6** and **7**), and **4**, which bears both the galloyl and the hexahy-

droxydiphenoyl (HHDP) groups. Among the active compounds, only the 2,3- and 4,6-coupled ellagitannin, pedunculagin (**2**), showed competitive inhibition on thrombin. The hydroxyl groups of the sugar moiety are highly acylated by either galloyl or HHDP groups in these active compounds. In contrast, compound **3**, having only two hydroxyl groups acylated by two galloyl groups at C-2,C-6, and compound **5**, with one HHDP group at C-2, C-3, are inactive. Similarly, compounds **1a**, **1b**, and **2a**, bearing no phenyl hydroxyl groups, but with methylated hydroxyl groups, also showed no measurable inhibition on thrombin at a higher concentration of 100 μM. It is indicated that phenolic hydroxyl groups of the active tannins play an important role in their inhibitory activity on thrombin. From a pharmaceutical point of view, compound **2** can be considered as a good direct thrombin inhibitor for controlling thrombosis because of its potent anticoagulant activity, relative selectivity, and lower toxicity, with an LD_{50} of >100 mg/kg p.o. in mice and rats.[17] The total synthesis of compound **2** has recently been reported.[18] Although the bioactivities of tannins have been studied extensively,[19] this is the first report of the effects of tannins from a plant source on the enzymes of blood coagulation cascade. Further work on the selective effects of other serine proteinases and on the effects of the blood coagulation system in vivo should be evaluated individually.

Experimental Section

General Experimental Procedures. ESIMS were collected on a PE SCIEX API-300 mass spectrometer. All spectra (^{1}H NMR, ^{13}C NMR, COSY, HMQC, and HMBC) were recorded on a Bruker AMX 500 spectrometer (500 MHz for ^{1}H and 125 MHz for ^{13}C), and the chemical shifts are reported in parts per million using TMS as an internal standard. TLC was done on HPTLC−Fertigplaten Cellulose F (Merck) plates in the solvent systems: (a) H_2O and (b) 7% aqueous HOAc.

Material and Reagents. The plant of *Geum japonicum* was collected from Guizhou Province of the People's Republic of China in August 1994. It was identified by Dr. Dao-Feng Chen, Department of Pharmacognosy, Shanghai Medical University, People's Republic of China. A voucher specimen is deposited in the Department of Pharmacognosy, Shanghai University of Traditional Chinese Medicine, People's Republic of China.

Heparin sodium salt (from porcine intestinal mucosa, 187 USP units/mg), thromboplastin with calcium reagent (for PT test), TT reagent, APTT reagent, thrombin from bovine plasma (3 NIH units/vial), and fibrinogen, fraction I, type IV, from bovine plasma were purchased from Sigma Chemical Co. (St. Louis, MO). Human Factor Xa was purchased from ICN Biomedicals Inc. (Aurora, OH). Substrates S-2238 (H-D-Phe-Pip-Arg-*p*-nitroanilide) and S-2222 (*N*-benzoyl-L-isoleucyl-L-arginine-*p*-nitroaniline hydrochloride and its methyl ester) were obtained from Chromogenix Co. (Mölndal, Sweden). (Sweden).

Extraction and Isolation. Dried whole plant (2.6 kg) was chopped into small pieces and percolated three times with MeOH (20 L) at room temperature, and the extract was evaporated in vacuo to yield the MeOH dried extract (350 g). This latter was resuspended in distilled H_2O (1 L) and successively partitioned with hexane (2 L (5), EtOAc (2 L × 5) and *n*-BuOH (1 L × 5). The *n*-BuOH-soluble fraction was filtered, and the filtrate evaporated under reduced pressure (50 °C) to give a brown residue (73 g). The *n*-BuOH extract was subjected to Sephadex LH-20 column chromatography and eluted with H_2O−MeOH starting with H_2O (increasing amount of MeOH), giving nine fractions, fractions 1−9. Fraction 9, eluted with 80% MeOH (3.3 g), showed potent anticoagulant activity. It was further chromatographed over Sephadex LH-20, eluted with the above solvent system, to give three fractions, I (0.08 g), II (1.7 g), and III (1.2 g). A combination of column chromatography on Sephadex LH-20 and cellulose of fraction II, eluted with 50% MeOH, using $CHCl_3$−MeOH (1:1), MeOH, 3% HOAc as eluents, followed by purification on Bio-Gel P-4 (extra fine, <45 μM) column with 0.5% HOAc, results in the isolation of compounds **2** (60.8 mg), **3** (36.2 mg), **5** (9.6 mg), **6** (8.1 mg), and **7** (10.2 mg). Fraction III, eluted with MeOH, was chromatographed over cellulose with MeOH−HOAc−H_2O (5:5:90) and then over Toyopearl HW-40s with 30% aqueous MeOH to afford **1** (120 mg) and **4** (9 mg).

Of these compounds, the assignments of the ^{1}H or ^{13}C NMR spectral data of the sugar moieties of **6**[8,20] and **7**[12] were different from the data reported in the literature. The ^{1}H and ^{13}C NMR data of the sugar moiety were reassigned with the help of DEPT, ^{1}H−^{1}H COSY, HMQC, and HMBC spectra data. The ^{13}C NMR data of the sugar moieties of **6**: (CD_3COCD_3, 175 MHz,) δ 66.1 (C-1), 81.7 (C-2), 73.8 (C-3), 77.0 (C-4), 69.6 (C-5), 68.7 (C-6); and **7**: (CD_3COCD_3, 175 MHz) δ 68.8 (C-1), 77.5 (C-2), 71.6 (C-3), 78.0 (C-4), 69.2 (C-5), 68.9 (C-6). ^{1}H NMR data of **7**: (CD_3COCD_3, 500 MHz) δ 5.64 (1H, d, J = 4.8 Hz, H-1), 5.45 (1H, t, J = 2.5 Hz, H-3), 5.01 (1H, dd, J = 2.8, 8.6 Hz, H-4), 4.72 (1H, dd, J = 2.3, 4.8 Hz, H-2), 4.64 (1H, dd, J = 3.2, 12.3 Hz, H-6), 4.12 (1H, dd, J = 2.5, 8.6 Hz, H-5), 3.83 (1H, d, J = 11.8 Hz, H-6).

Synthesis of Penta-*O*-benzoyl-β-D-glucopyranoside (1a). A solution of 1 g (5.6 mmol) of D(+)-glucopyranoside, 3.6 g (29.5 mmol) of benzoic acid, 0.33 g (2.7 mmol) of DMAP (4-dimethylaminopyridine), 0.41 g (2.7 mmol) of DMAP·HCl, and 3.7 g (17.9 mmol) of DCC (1,3-dicyclohexylcarbodiimide) in 100 mL of CH_2Cl_2 was purged under N_2 and then refluxed for 24 h. The reaction mixture was cooled to room temperature and filtered. Solvent was removed to give a white foam. The crude product was purified by chromatography on Si gel (CH_2Cl_2) to afford 1.5 g of penta-*O*-benzyol-β-D-glucopyranoside (**1a**). ESIMS: 723.5 $[M + Na]^+$; ^{1}H NMR ($CDCl_3$, 500 MHz) δ 6.34 (1H, d, J = 8.4 Hz, Glu H-1), 6.09 (1H, t, J = 9.4 Hz, Glu H-3), 5.90 (1H, t, J = 9.8 Hz, Glu H-4), 5.73 (1H, dd, J = 3.7, 10.2 Hz, Glu H-2), 4.43−4.68 (3H, m, Glu H-5, 6), 7.31−8.07 (25H, m, aromatic protons).

Methylation of 1. An EtOH solution (0.5 mL) of **1** (5 mg) was treated with ethereal CH_2N_2 (1.5 mL) for 1 h at room temperature. The solvent was evaporated off, and the residue was subjected to preparative TLC (CH_2Cl_2−CH_3COCH_3, 7:3) to give a pentadecamethylate (**1b**) (2.1 mg, R_f = 0.6). ESIMS: 1173.5 $[M + Na]^+$; ^{1}H NMR (CD_3COCD_3, 500 MHz) δ 7.36 (2H, s, galloyl), 7.32 (2H, s, galloyl), 7.26 (2H, s, galloyl), 7.25 (2H, s, galloyl), 7.21 (2H, s, galloyl), 6.41 (1H, d, J = 8.1 Hz, Glu H-1), 6.21 (1H, t, J = 9.7 Hz, Glu H-3), 5.90 (1H, t, J = 9.7 Hz, Glu H-4), 5.78 (1H, dd, J = 8.1, 9.4 Hz, Glu H-2), 4.87 (1H, dd, J = 3.1, 12.1 Hz, Glu H-6), 4.74 (1H, m, Glu H-5), 4.44 (1H, dd, J = 5.3, 12.1 Hz, Glu H-6), 3.91 (9H, 3 × OCH_3), 3.89 (6H, 2 × OCH_3), 3.88 (3H, OCH_3), 3.83 (6H, 2 × OCH_3), 3.82 (9H, 2 × OCH_3), 3.80 (3H, OCH_3), 3.79 (3H, OCH_3), 3.76 (3H, OCH_3), 3.73 (3H, OCH_3), and 3.72 (3H, OCH_3).

Methylation of 2. An EtOH solution (0.5 mL) of **1** (6 mg) was treated with ethereal CH_2N_2 (1.5 mL) for 2 h at room temperature. The solvent was evaporated off, and the residue was subjected to preparative TLC (CH_2Cl_2−CH_3COCH_3 7:3) to give an isomer mixture of trideca-*O*-methylpedunculagin (**2a**) (1.6 mg). ESIMS: 989.5 $[M + Na]^+$; ^{1}H NMR (CD_3COCD_3, 500 MHz) δ 6.96 (1H, s, HHDP), 6.93 (1H, s, HHDP), 6.89 (1H, s, HHDP), 6.85 (2H, s, HHDP), 6.81 (1H, s, HHDP), 6.62 (1H, s, HHDP), 6.60 (1H, s, HHDP), 5.53 (1H, t, J = 10.5 Hz, Glu H-3), 5.28 (1H, t, J = 9.8 Hz, Glu H-3), 5.24−5.08 (5H, m, Glu H-1α, 2α, 2β, 6 × 2), 5.02 (1H, t, J = 10.1 Hz, Glu H-4), 4.85 (1H, t, J = 8.7 Hz, Glu H-4), 4.88 (1H, d, J = 6.4 Hz, Glu H-1β), 4.65 (1H, m, Glu H-5), 4.29 (1H, m, Glu H-5), 4.13 (2H, m, Glu H-6), 3.61−3.93 (78H, m, 26 × OCH_3)

Biological Assays. Blood-clotting assays:[21] The effects of these compounds on coagulation of rabbit plasma in vitro were measured at 37 °C on a fibrometer (BBL FibroSystem, Becton Dickinson Co., Cockeysville, MD) using the PT assay; 150 μL of 0.05 M Tris buffer containing 0.1 M NaCl (pH 7.5), 50 μL of sample solution (compound dissovled in 0.05 M Tris buffer), and 100 μL rabbit plasma were incubated for 2 min at 37 °C. Clotting was initiated by addition of 100 μL of

thromboplastin with calcium reagent (Sigma). Similarly, the TTs were measured by addition of 100 μL bovine thrombin (Sigma, 3−4 NIH units/mL) to a mixture of rabbit plasma (100 μL), 0.05 M Tris buffer (50 μL), and compounds dissolved in Tris buffer (50 μL), which was preincubated for 2 min. APTT was measured by incubating rabbit plasma (100 μL), 0.05 M Tris buffer (50 μL), and compounds dissolved in Tris buffer (50 μL) with APTT reagent (100 μL) for 2 min. Clotting was initiated by adding 25 mM $CaCl_2$ (100 μL). Clotting times are based on the average of three separate determinations.

Determination of the inhibitory activity of tannins for thrombin:[13] The activity of thrombin from bovine plasma was measured using S-2238 as substrate on a Ceres 900C Microplate Autoreader (Bio-Tek Instruments, Inc., Winooski, VT). The assay mixture of 200 μL comprises 10 μL thrombin (0.19 NIH units/mL) in 0.05 M Tris buffer containing 0.1 M NaCl (pH 7.5) and 10 μL of various concentrations of compounds, which was preincubated with thrombin for 2 min at 37 °C. The reaction was started by the addition of 20 μL S-2238 (0.66 mM). Activity against the substrate was determined by measurement of the release of *p*-nitroaniline from S-2238 as indicated by the increase in optical density at 405 nm over 5 min. The results were expressed as percentage of inhibition of thrombin activity. The kinetics of thrombin from bovine plasma were studied, with a range of substrate concentrations from 0.6 to 1.7 mM.[22] Assays were performed by using a 0.05 M Tris buffer containing 0.1 M NaCl (pH 7.5) and a fixed concentration of thrombin from bovine plasma (0.19 NIH units/mL) at 37 °C. The total volume of reaction mixture was maintained at 200 μL. Reactions were initiated by addition of substrate to the cuvette containing thrombin and compound preincubated for less than 2 min. Data from initial rate experiments were used to construct Lineweaver−Burk plots,[23] while the relationship of (substrate concentration)$^{-1}$ versus (initial velocity)$^{-1}$ was analyzed by linear regression. The type of inhibition and K_i values were determined from the plots.[24] Compounds **1a**, **1b**, and **2a** were tested in 0.1% DMSO in Tris buffer containing 0.1 M NaCl (pH 7.5), which was used as control.

Thrombin-induced fibrin-clot assays:[25] All solutions used were prepared with 0.05 M Tris buffer containing 0.1 M NaCl (pH 7.5), except for fibrinogen, which was reconstituted in ultrapure H_2O. For the assay, 190 μL of different concentrations of a compound were preincubated for 3 min with 10 μL of thrombin (0.05 NIH units/mL) at 37 °C, and then 50 μL fibrinogen (2 mg/mL) were added to initiate enzymatic reaction. The turbidity of clots was measured for 5 min at 405 nm, and velocities were calculated as milli-optical density units per minute (mOD/min). Each assay was performed in triplicate. The percentage of inhibition of thrombin-induced fibrin clotting was calculated according to the formula: percentage inhibition (%) = [1 − (velocity of samples/velocity of control)] × 100.

Determination of the inhibitory activity of tannins for factor Xa:[26] The activity of human factor Xa was measured with S-2222 as the substrate. The assay mixtures of 200 μL contained 0.20 mM S-2222 in 0.05 M Tris buffer containing 0.1 M NaCl (pH 8.3) and various concentrations of tannins. The reaction was started by addition of factor Xa (50 ng/mL). Activity against the substrate was determined by measurement of the release of 4-nitroanilide as indicated by the increase in optical density at 405 nm over 5 min at 37 °C. For determination of the K_i, increasing concentrations of S-2222 were added (0.04 mM−0.20 mM). The IC_{50} value was the concentration inhibiting 50% of the initial activity of enzymes. The data were collected as mean ± SD of three separate experiments.

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