Structure of an Anti-plasmin Inhibitor, Eckol, Isolated from the Brown Alga *Ecklonia kurome* OKAMURA and Inhibitory Activities of Its Derivatives on Plasma Plasmin Inhibitors[1,2]

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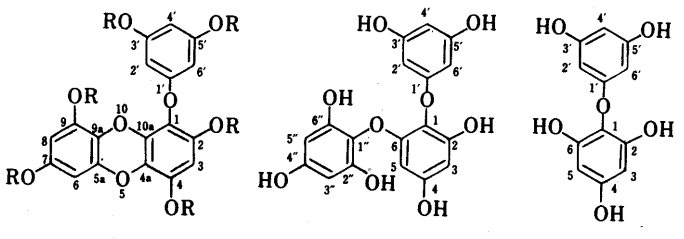
Eckol (1), a novel phlorotannin with a dibenzo-1,4-dioxin skeleton, has been isolated from the brown alga *Ecklonia kurome* OKAMURA as a potent and specific anti-plasmin inhibitor. Its structure has been elucidated based on the spectral data, in particular, by means of negative nuclear Overhauser effect (NOE), and finally established as 1-(3,5-dihydroxyphenoxy)-2,4,7,9-tetrahydroxydibenzo-1,4-dioxin by X-ray analysis. Some partially methoxylated derivatives of eckol were prepared by methylation with diazomethane and also by selective demethylation of eckol permethylate (1b) to establish the structural requirements for inhibitory activities on α_2-macroglobulin and α_2-plasmin inhibitor, the main plasmin inhibitors in plasma.

Keywords eckol; polyphenol; phlorotannin; *Ecklonia kurome*; negative NOE; X-ray analysis; anti-plasmin inhibitor; α_2-macroglobulin; α_2-plasmin inhibitor; fibrinolytic activity

Plasmin plays an important role in the fibrinolytic enzyme system and is well known to degrade the insoluble fibrin.[4] Thus, plasminogen activators such as urokinase and streptokinase, have been used clinically for thrombolytic therapy over the past decade. However, activated plasmin thereby generated is trapped instantaneously by plasmin inhibitors, *i.e.* α_2-macroglobulin (α-M) and α_2-plasmin inhibitor (α-PI), and hence little effect on thrombosis can be expected.[5] In addition, the effect of physiologically released plasminogen activator is reduced by these inhibitors in the blood. Considering the suppressing control in the fibrinolytic enzyme system, specific inhibitors of the plasma plasmin inhibitors (anti-plasmin inhibitors) may be useful in prevention and treatment of thrombosis. Along this line, we have been searching for potent and specific anti-plasmin inhibitors among natural products, and have already reported some inhibitors produced by microorganisms.[6,7] These inhibitors, however, were insufficiently specific.

In this paper, we wish to report on the structure of a potent and specific anti-plasmin inhibitor, eckol (**1**), belonging to a new class of phlorotannins,[8] which was isolated from the brown alga *Ecklonia kurome* OKAMURA. The structure–activity relationship is discussed.

Isolation and Structure of Eckol (1) The methanol extract of *E. kurome* collected in Kochi has been found to inhibit the action of α-M and α-PI, the main plasmin inhibitors in plasma. Bioassay-directed fractionation led to the isolation of a new type of phlorotannin bearing a dibenzo-1,4-dioxin skeleton, designated as eckol.[9]



1: R=H
1a: R=Ac
1b: R=CH_3

2

3

Chart 1

Eckol (**1**), mp 243—244 °C, has the molecular formula $C_{18}H_{12}O_9$ (*m/z* 372.0460; Calcd 372.0481) indicating thirteen degree of unsaturation. The infrared (IR) spectrum of **1** revealed the presence of a hydroxy group (3250 cm^{-1}) and an aromatic nucleus (1605 cm^{-1}), but had no carbonyl absorption. The carbon-13 nuclear magnetic resonance (^{13}C-NMR) spectrum of **1** indicated the presence of six non-substituted and twelve O-bearing aromatic carbons (Table I), whereas the proton nuclear magnetic resonance

TABLE I. ^{13}C-NMR Spectra Data[a] for **1**,[b] **1b**,[c] **2**,[b] and **3**[b]

Carbon	1	1b	Carbon	2	3
C-1	123.5 s	126.1 s	C-1	123.4 s	122.9 s
C-2	145.9 s	148.81 s	C-2	151.2 s	151.3 s
C-3	98.4 d	93.5 d	C-3	95.9 d	95.0 d
C-4	141.9 s	144.6 s	C-4	154.3 s	154.6 s
C-4a	122.6 s	126.8 s	C-5	93.0 d	95.0 d
			C-6	153.1 s	151.3 s
C-5a	142.7 s	142.7 s	C-1′	160.8 s	160.8 s
C-6	94.0 d	94.2 d	C-2′	94.2 d	93.9 d
			C-3′	158.8 s	158.8 s
			C-4′	95.9 d	95.8 d
C-7	153.0 s	155.8 s	C-5′	158.8 s	158.8 s
			C-6′	94.2 d	93.9 d
C-8	98.7 d	96.5 d			
C-9	146.1 s	148.84 s			
C-9a	122.9 s	125.8 s	C-1″	122.5 s	
C-10a	137.3 s	137.6 s	C-2″	151.2 s	
C-1′	160.4 s	160.7 s	C-3″	95.0 d	
C-2′	94.0 d	94.5 d	C-4″	154.6 d	
C-3′	158.7 s	161.4 s	C-5″	95.0 d	
C-4′	96.4 d	94.4 d	C-6″	151.2 s	
C-5′	158.7 s	161.4 s			
C-6′	94.0 d	94.5 d			
C_2-OCH_3		57.0 q			
C_4-OCH_3		56.8 q			
C_7-OCH_3		55.6 q			
C_9-OCH_3		56.8 q			
$C_{3'}$-OCH_3		55.4 q			
$C_{5'}$-OCH_3		55.4 q			

a) Assignments were confirmed by long-range selective proton decoupling (LSPD). *b*) In DMSO-d_6. *c*) In $CDCl_3$.

(^{1}H-NMR) spectrum contained signals characteristic of six aromatic protons, *viz.* an AB_2 system at δ 5.83 (1H, J= 2.2 Hz) and 5.75 (2H, J=2.2 Hz), an AB system at δ 5.82 and 5.98 (J=2.7 Hz), and a singlet at δ 6.16 (1H) as well as six phenolic hydroxy protons at δ 9.17 (2H), 9.19, 9.20, 9.48, and 9.53. These NMR spectral features are very similar to those of triphloroethol (**2**)[10] isolated from *Laminaria ochroleuca*, indicating that **1** is composed of three phloroglucinol units. The only difference between the ^{1}H-NMR spectra of **1** and **2** is that the former lacks the signals for one phenolic hydroxy proton and one aromatic proton, suggesting that **1** has an additional aryl-ether linkage. This was supported by the presence of a new oxygen-bearing carbon signal (δ 122.6), which is characteristic of an aromatic carbon with two oxygenated neighbors, and also by the formation of a hexaacetate (**1a**) and a hexamethylate (**1b**) on usual acetylation and permethylation (NaH–dimethylformamide (DMF)–CH_3I), respectively. The complete assignment of all the proton signals in the ^{1}H-NMR spectrum of **1** was accomplished on the basis of the negative nuclear Overhauser effect (NOE) experiments (−30——40%) (Fig. 1).[11] Namely, selective irradiations of the phenolic hydroxy protons, $H_{e,f}$ (δ 9.17) led to reductions in the intensities of the aromatic resonances H_i (δ 5.83) and $H_{l,k}$ (δ 5.75) indicating the presence of the partial structure A. Since both H_h (δ 5.98) and H_j (δ 5.82) aromatic resonances were reduced in intensity upon irradiation of H_c (δ 9.20) and also irradiation of H_a (δ 9.53) led to a decrease in the intensity of the H_h signal, H_h and H_j had to be located *ortho* to both the OH_a and OH_c groups, and to the OH_c group, respectively, indicating the presence of another partial structure B. Similarly, the partial structure C was derived from the distinct negative NOEs (H_g) observed upon selective irradiation of the phenolic hydroxy protons H_b (δ 9.48) and H_d (δ 9.19). The negative NOEs in **1** were consistent with those in the methylate (**1b**). Thus, the linkage of the partial structures A, B, and C, together with biosynthetic considerations, resulted in the proposal of the structure (**1**) with a dibenzo-1,4-dioxin unit.

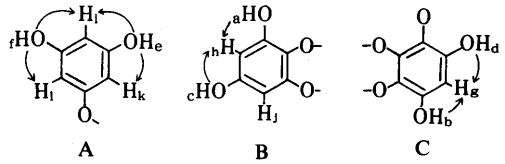


Fig. 1. Partial Structures of Eckol (**1**)
The observed negative NOEs are indicated by arrows.

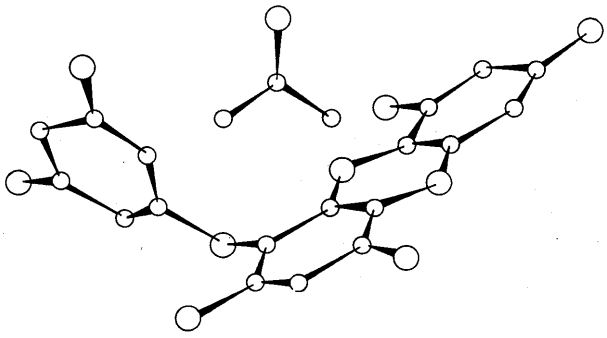
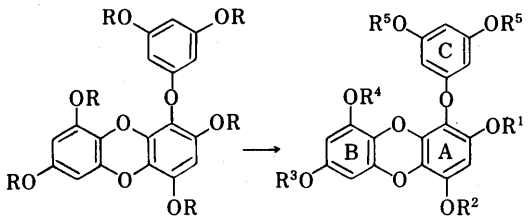


Fig. 2. Perspective Drawing of the Molecule of **1**

Although the tentative structure was plausible, an alternative dibenzo-1,4-dioxin ring derived from B and C could not be ruled out. This problem was settled unambiguously by an X-ray analysis of a single crystal of **1** grown up in acetone–water. The perspective drawing of **1**, including a hydrogen-bonded acetone molecule is shown in Fig. 2. Accordingly, the structure of eckol was established as **1**.

Syntheses of Various Methylated Derivatives of Eckol and Their Inhibitory Activities on Anti-plasmins Eckol, which is made up from three units of phloroglucinol presumably *via* an oxidative coupling, can be regarded as a new type of phlorotannin[12] having a dibenzo-1,4-dioxin skeleton. This unique substance exhibited potent and specific inhibitory activities on the actions of α-M (IC_{50} 2.5 μg/ml) and α-PI (IC_{50} 1.60 μg/ml) without affecting plasma proteases such as trypsin, plasmin, and urokinase at as high a concentration as 100 μg/ml. On the other hand, phloroglucinol itself, triphloroethol (**2**), and diphloroethol (**3**)[10] did not show inhibitory activity at all, revealing the requirement of a 1,4-dioxane ring for anti-plasmin inhibitory activity. It is necessary to clarify the contributions of various structures to the inhibitory activity of **1** against plasmin inhibitors in order to obtain more potent derivatives of the title compound. Hence, we have tried to methylate selectively the six hydroxy groups existing in **1**. The result of the X-ray analysis of **1** was valuable in connection with this. Namely, the C_2-OH (1.367 Å) and C_4-OH (1.369 Å) bond lengths were found to be shorter than the other C-OH bonds, suggesting that both OH groups are more acidic than the other OH ones. Thus, they can be discriminated in terms of reactivity.

Treatment of **1** with ethereal diazomethane yielded **1c**, **1d**, and **1e** which were methylated at the more acidic C_2-OH and C_4-OH, the quantitative ratio depending on the reaction period. As anticipated, the yields of compounds methylated at the C_4-OH and $C_{3',5'}$-OH were so small that these compounds could not be isolated. Moreover, **1** could be selectively benzylated at C_2-OH and C_4-OH with *n*-BuLi at −78 °C followed by the addition of benzyl bromide. The resulting dibenzyl compound was completely methylated (NaH–DMF–CH_3I) and then subjected to hydrogenolysis



1: R=H
1b: R=CH_3

	R^1	R^2	R^3	R^4	R^5
1c	CH_3	CH_3	H	CH_3	H
1d	H	CH_3	H	CH_3	H
1e	H	H	H	CH_3	H
1f	H	H	CH_3	CH_3	CH_3
1g	CH_3	CH_3	CH_3	H	CH_3
1h	H	CH_3	CH_3	H	CH_3
1i	H	H	H	H	CH_3
1j	H	H	CH_3	H	H
1k	H	CH_3	H	H	CH_3
1l	CH_3	H	H	H	CH_3
1m	H	H	CH_3	H	CH_3

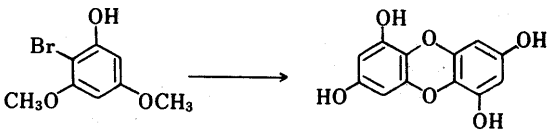
Chart 2

over 10% Pd–C, affording 3′,5′,7,9-tetramethylated eckol (**1f**).

Next, our efforts were concentrated on selective demethylation of the permethylated eckol (**1b**) prepared from K_2CO_3–CH_3I–DMF. Treatment of **1b** with BCl_3 at room temperature afforded compounds **1g** and **1h** demethylated at C_2-OCH_3 and C_9-OCH_3, respectively, due to a chelation effect between the neighboring oxygens and the reagent. On the other hand, **1b** was completely demethylated at room temperature with BBr_3. The same reaction, however, was employed at 0 °C to afford 3′,6′-dimethoxy and 7-methoxy eckols (**1i** and **1j**), respectively, and at −20 °C to yield the trimethylated compounds, **1k**, **1l**, and **1m**.

Finally, dibenzo-1,4-dioxin-1,3,6,8-tetraol (**5**) regarded as the basic skeleton of eckol, was synthesized starting from 4,5-dimethoxyphenol by means of the Ullman reaction.

Speaking generally, the position of a methoxy group is



4 5

Chart 3

TABLE II. ^{1}H-NMR Chemical Shifts for the OCH_3 Protons of Eckol Permethylate (**1b**) and Its Derivatives[d]

Compound	2-OCH_3	4-OCH_3	7-OCH_3	9-OCH_3	3′,5′-OCH_3
1b[a]	3.77	3.91	3.71	3.66	3.71
1c[a]	3.75	3.89		3.65	
1d[b]		3.76		3.62	
1e[b]				3.62	
1f[a]			3.73	3.65	3.75
1g[a]	3.80	3.93	3.69		3.75
1h[a]		3.89	3.69		3.75
1i[b]					3.68
1j[c]			3.71		
1k[a]		3.88			3.75
1l[a]	3.78				3.75
1m[a]			3.70		3.75

a) In $CDCl_3$. *b*) In DMSO-d_6. *c*) In $CDCl_3$ + 10% DMSO-d_6. *d*) The position of each OCH_3 group was confirmed by NOE and acetylation.

TABLE III. Inhibitory Activities (IC_{50}, μg/ml) of Eckol (**1**) and Its Derivatives on α_2-Macroglobulin (α-M) and α_2-Plasmin Inhibitor (α-PI)

Compound	α-M	α-PI
1	2.5	1.60
1b	>100	>100
1g	>100	>100
1f	>30[a]	>100
1h	>100	>100
1c	>30[b]	>100
1d	>100	72
1i	6.6	0.52
1l	7.4	1.40
1k	5.8	0.30
1m	3.3	0.70
1e	1.7	0.45
1j	2.8	0.71
5	7.0	1.40

a) 21% inhibition at 30 μg/ml. *b*) 3% inhibition at 30 μg/ml.

difficult to assign without the aid of an NOE experiment. However, the chemical shift values corresponding to the OCH_3 protons in **1b** allow the formation of a general rule for the assignment of methoxy groups in the derivatives of **1** as follows (see Table II): among the six OCH_3, 1) the signal due to the C_4-OCH_3 appears at the lowest field (δ 3.91), 2) the signal for C_9-OCH_3 is shifted to the highest field (δ 3.66) due to an anisotropy effect of ring C, 3) the three signals due to the remaining four OCH_3 are located intermediately, but the chemical shifts for C_2-OCH_3 and C_7-OCH_3 appear at lower field and at higher field than that of the six H integrated $C_{3',5'}$-OCH_3. According to the above general rule, the position methylated in each derivative can be readily determined as shown in Table II.

The resulting derivatives were subjected to screening for inhibitory activity against α-M and α-PI. According to the assay results shown in Table III, the $C_{3',5'}$-OH groups on the C ring appear not to be essential for the inhibitory activity (see **1i**—**1m**), and a role of the C ring itself can be excluded since compound **5**, a key dibenzo-1,4-dioxin with no dihydroxyphenoxy moiety, exhibited strong inhibitory activities on both α-M and α-PI. In addition, it is worthy of note that at least three free hydroxy substituents on both the A and B rings are required for anti-plasmin inhibitory activity (see **1l**—**1j**) and in particular, the compound (**1m**) methylated as C_7-OH and $C_{3',5'}$-OH on the B and C rings, respectively, showed almost identical activities with those of eckol (**1**).

Eckol itself is considered to be a promising antithrombotic agent and a potentiator of thrombolytic enzymes, such as urokinase. The structure–activity relationships of its methylated derivatives imply that a simple dibenzo-1,4-dioxin skeleton bearing some functional groups may become a lead compound for the development of a new class of thrombolytic agents.[13]

Experimental

All melting points were determined on a Yanagimoto micro melting point apparatus and are uncorrected. Ultraviolet (UV) spectra were recorded on a Hitachi 340 spectrophotometer. IR spectra were measured with a Jasco A-202 spectrophotometer. ^{1}H- and ^{13}C-NMR spectra were obtained at 400 MHz (^{1}H-NMR) and 100.16 MHz (^{13}C-NMR) using a Bruker WH 400 spectrometer, respectively. Chemical shifts were expressed in (ppm) downfield from tetramethylsilane as an internal standard. The mass spectra (MS) were recorded on a Varian MAT 200. High performance liquid chromatography (HPLC) was performed by using a Waters 6000 A pump, a Jasco UVDEC-100-II UV detector, and a Hypersil ODS column (10 × 300 mm). Silica gel (Wako, C-300), Sephadex (Pharmacia Fine Chemicals, LH-20), and Bio-Bead (Bio-Red, SX-12) were used for column chromatography. Both Silica gel F_{254} and RP-8 F_{254} (Merck) were used for analytical thin layer chromatography, and spots were visualized by UV (254 nm) illumination and by spraying 40% $CeSO_4$–H_2SO_4 followed by heating.

Extraction and Purification of Eckol (1) Fresh whole plants (600 kg) of *Ecklonia kurome* OKAMURA collected in Irino, Kochi prefecture, were immersed in methanol at room temperature for 6 d. The methanol was evaporated *in vacuo* to give a gummy extract, which was partitioned between EtOAc and water. The EtOAc soluble portion (1.7 kg) mixed with celite (3.4 kg) was dried under reduced pressure. The obtained solids were pulverized, packed into a glass column, and eluted in order with benzene (18 l), methylene chloride (36 l), ether (54 l), and methanol (20 l). The fraction (552 g) eluted with ether was subjected to Sephadex LH-20 (3.5 kg) chromatography. Each fraction (2 l) eluted with acetone was collected, and the fourth fraction was evaporated *in vacuo* to yield eckol (**1**) (40 g) as crystals.

Eckol (1) Colorless plates (from acetone–H_2O), mp 243—244 °C. High-resolution MS: Found, *m/z* 372.0460 (M^+), Calcd for $C_{18}H_{12}O_9$, *m/z*

372.0481. UV λ_{max}^{MeOH} nm (ε): 230 (30000), 290 (3160). IR ν_{max}^{KBr} cm^{-1}: 3250, 1605, 1475, 1370, 1260, 1200, 1160,1140, 1110, 1083, 1040, 1010, 805. EI-MS *m/z* (rel. int.): 372 (M^+, 100), 264 (46), 232 (25). ^{1}H-NMR (DMSO-d_6) δ: 5.75 (2H, d, *J*=2.2 Hz), 5.82 (1H, d, *J*=2.7 Hz), 5.83 (1H, t, *J*=2.2 Hz), 5.98 (1H, d, *J*=2.7 Hz), 6.16 (1H, s), 9.17 (2H, s), 9.19 (1H, s), 9.20 (1H, s), 9.48 (1H, s), 9.53 (1H, s). ^{13}C-NMR: see Table I.

Eckol Hexaacetate (1a) A mixture of **1** (200 mg), acetic anhydride (1 ml), and pyridine (2 ml) was allowed to stand on at room temperature for 20 h. The reaction mixture was poured onto crushed ices and the precipitate was collected by filtration and recrystallized from benzene–methanol to afford **1a** (280 mg) as colorless prisms, mp 211—212.5 °C. IR ν_{max}^{KBr} cm^{-1}: 2900, 2850, 1750, 1595, 1360, 1175, 1120, 1100, 1010, 875. EI-MS *m/z* (rel. int.): 624 (M^+, 6), 582 (22), 540 (22), 498 (36), 456 (36), 418 (9), 372 (12), 43 (100). ^{1}H-NMR ($CDCl_3$) δ: 1.96 (3H, s), 2.13 (3H, s), 2.24 (3H, s), 2.26 (6H, s), 2.34 (3H, s), 6.50 (1H, d, *J*=2.6 Hz), 6.58 (2H, d, *J*=2.0 Hz), 6.63 (1H, d, *J*=2.6 Hz), 6.66 (1H, s), 6.73 (1H, t, *J*=2.0 Hz).

Eckol Hexamethylate (1b) A mixture of **1** (500 mg, 1.3 mmol), K_2CO_3 (1.5 g), and methyl iodide (3 ml) in anhydrous DMF (10 ml) was stirred at room temperature overnight. The reaction mixture was acidified with 1 N HCl and then extracted with EtOAc. The EtOAc was washed with brine, and dried over $MgSO_4$. The solvent was removed *in vacuo* to leave a red oil, which was chromatographed on silica gel (10 g) with benzene, giving **1b** as colorless prisms (510 mg) (from ether–methylene chloride), mp 171—172 °C. IR ν_{max}^{KBr}: 2925, 2830, 1590, 1500, 1450, 1370, 1200, 1110, 1050, 970, 910, 810. EI-MS *m/z* (rel. int.): 456 (M^+, 100). ^{1}H-NMR ($CDCl_3$) δ: 3.66 (3H, s), 3.71 (9H, s), 3.77 (3H, s), 3.91 (3H, s), 6.09 (1H, d, *J*=2.9 Hz), 6.13 (1H, t, *J*=2.1 Hz), 6.17 (2H, d, *J*=2.1 Hz), 6.26 (1H, s). ^{13}C-NMR: see Table I.

Methylation of 1 with Diazomethane An etheral solution prepared from nitrosomethylurea (200 mg) was added to a solution of **1** (100 mg, 0.26 mmol) in MeOH (5 ml) at 0 °C. The reaction mixture was allowed to stand at room temperature overnight. The solvent was evaporated off *in vacuo* to leave a viscous oil, which was chromatographed over silica gel (10 g) with MeOH–$CHCl_3$ (1 : 9). Compounds **1c** (30 mg), **1d** (43 mg), and **1e** (30 mg) were eluted in order. **1c**: Colorless needles (from $CHCl_3$–MeOH), mp 267—268 °C. UV λ_{max}^{MeOH} nm (ε): 232 (54000), 290 (3300). EI-MS *m/z* (rel. int.): 414 (M^+, 100), 282 (2), 315 (12), 260 (10). ^{1}H-NMR ($CDCl_3$–10% DMSO-d_6) δ: 3.65 (3H, s), 3.75 (3H, s), 3.89 (3H, s), 6.00 (2H, d, *J*=2.0 Hz), 6.05 (1H, t, *J*=2.0 Hz), 6.07 (1H, d, *J*=2.6 Hz), 6.15 (1H, d, *J*=2.6 Hz), 6.24 (1H, s), 8.25 (2H, s), 8.60 (1H, s). **1d**: Colorless prisms (from $CHCl_3$–MeOH), mp 287—288 °C. EI-MS *m/z* (rel. int.): 400 (M^+, 100), 367 (6), 276 (17), 246 (22). ^{1}H-NMR (DMSO-d_6) δ: 3.62 (3H, s), 3.76 (3H, s), 5.74 (2H, d, *J*=2.2 Hz), 5.83 (1H, t, *J*=2.2 Hz), 5.96 (1H, d, *J*=2.4 Hz), 6.08 (1H, d, *J*=2.4 Hz), 6.29 (1H, s), 9.18 (2H, s), 9.45 (2H, s). **1e**: Colorless prisms (from MeOH–water), mp 196—197 °C. UV λ_{max}^{MeOH} nm (ε): 232 (29500), 292 (2800). EI-MS *m/z* (rel. int.): 386 (M^+, 100). ^{1}H-NMR (DMSO-d_6) δ: 3.62 (3H, s), 5.73 (2H, d, *J*=2.0 Hz), 5.81 (1H, t, *J*=2.0 Hz), 5.96 (1H, d, *J*=2.7 Hz), 6.07 (1H, d, *J*=2.7 Hz), 6.16 (1H, s), 9.15 (2H, s), 9.24 (1H, s), 9.43 (1H, s), 9.50 (1H, s).

3',5',7,9-Tetramethoxyeckol (1f) A 1.39 M solution of *n*-BuLi in hexane (0.7 ml, 0.96 mmol) was added *via* a syringe to a solution of **1** (300 mg, 0.8 mmol) in tetrahydrofuran (THF) (10 ml)–hexamethylphosphoramide (HMPA) (3 ml) at −78 °C under an N_2 atmosphere. The reaction mixture was stirred for 10 min, then a solution of benzyl bromide (164 mg, 0.96 mmol) in THF (1 ml) was added. Stirring was continued at −78 °C for 30 min at 0 °C for 1 h. To this solution, a 50% oil dispersion of NaH (200 mg, 4 mmol) was added at 0 °C. After stirring at 0 °C for 10 min, methyl iodide (0.8 ml, 8 mmol) was added in one portion and then stirring was continued at room temperature for 5 h. The reaction mixture was quenched by the addition of ice-water and extracted with ether. The ether solution was washed with water and brine, dried over $MgSO_4$, and evaporated *in vacuo* to leave an oil, which was dissolved in EtOH (10 ml)–EtOAc (5 ml)–AcOH (3 drops). The resulting solution was hydrogenated over 10% Pd–C (20 mg) for 12 h. The catalyst was filtered off, and the filtrate was evaporated *in vacuo* to give an oil, which was purified by Bio-Bead chromatography with benzene to yield **1f** (60 mg) as a colorless amorphous substance. EI-MS *m/z* (rel. int.): 428 (M^+, 100). ^{1}H-NMR ($CDCl_3$) δ: 3.65 (3H, s), 3.73 (3H, s), 3.75 (6H, s), 5.15 (1H, s, OH), 5.21 (1H, s, OH), 6.10 (1H, d, *J*=2.7 Hz), 6.11 (1H, d, *J*=2.7 Hz), 6.17 (1H, t, *J*=2.1 Hz), 6.21 (2H, d, *J*=2.1 Hz), 6.31 (1H, d).

2,3',4,5',7-Pentamethoxyeckol (1g) and 3',4,5',7-Tetramethoxyeckol (1h) A solution (0.26 ml, 4 mmol) of 1.55 M BCl_3 in CH_2Cl_2 was added to a solution of **1b** (60 mg, 0.13 mmol) in CH_2Cl_2 (2 ml) at −78 °C. The reaction mixture was stirred at −78 °C for 3 h and at room temperature for 3 h, then acidified with 2 N HCl and extracted with CH_2Cl_2. The CH_2Cl_2 solution was concentrated *in vacuo* to afford an oil (50 mg), which was purified on a Lober column (Lichroprep Si-60, Type A) with $CHCl_3$–EtOAc (9 : 1) to yield **1g** (30 mg) as colorless prisms and **1h** (15 mg) as an amorphous substance. **1g**: mp 196—197 °C. High-resolution MS: Found, *m/z* 442.1260; Calcd for $C_{23}H_{22}O_9$, *m/z* 442.1264. EI-MS *m/z* (rel. int.): 442 (M^+, 100), 288 (10). ^{1}H-NMR ($CDCl_3$) δ: 3.69 (3H, s), 3.75 (6H, s), 3.80 (3H, s), 3.93 (3H, s), 4.93 (1H, s, OH), 6.12 (1H, d, *J*=2.8 Hz), 6.13 (2H, d, *J*=2.2 Hz), 6.16 (1H, t, *J*=2.2 Hz), 6.17 (1H, d, *J*=2.8 Hz), 6.29 (1H, s). **1h**: EI-MS *m/z* (rel. int.): 428 (M^+, 100). ^{1}H-NMR ($CDCl_3$) δ: 3.69 (3H, s), 3.76 (6H, s), 3.89 (3H, s), 4.78 (1H, s, OH), 5.22 (1H, s, OH), 6.11 (1H, d, *J*=2.8 Hz), 6.16 (1H, d, *J*=2.8 Hz), 6.18 (2H, d, *J*=2.2 Hz), 6.20 (1H, t, *J*=2.2 Hz), 6.35 (1H, s).

3',5'-Dimethoxyeckol (1i) and 7-Methoxyeckol (1j) A solution (1.57 ml, 1.31 mmol) of 0.84 M BBr_3 (4 ml) in CH_2Cl_2 was added dropwise to a solution of **1b** (100 mg, 0.219 mmol) in CH_2Cl_2 (4 ml) at 0 °C. The reaction mixture was stirred at 0 °C for 1 h and then quenched with water, acidified with 2 N HCl, and extracted with EtOAc. The EtOAc solution was evaporated *in vacuo* to afford an oil (70 mg), which was chromatographed on silica gel (5 g) with MeOH–CH_2Cl_2 to yield **1i** (27 mg) and **1h** (25 mg) as amorphous powders. **1i**: UV λ_{max}^{MeOH} nm (ε): 230 (29400), 292 (2900). MS *m/z* (rel. int.): 400 (M^+, 100), 287 (8), 260 (25), 245 (18). ^{1}H-NMR (DMSO-d_6) δ: 3.68 (6H, s), 5.80 (1H, d, *J*=2.7 Hz), 5.90 (1H, d, *J*=2.7 Hz), 6.00 (2H, d, *J*=2.2 Hz), 6.14 (1H, t, *J*=2.2 Hz), 6.16 (1H, s), 9.19 (1H, s), 9.27 (1H, s), 9.50 (1H, s), 9.51 (1H, s). **1j**: UV λ_{max}^{MeOH} nm (ε): 230 (36000), 292 (3400). EI-MS *m/z* (rel. int.): 386 (M^+, 100), 287 (5), 246 (40), 231 (25), 123 (30). ^{1}H-NMR ($CDCl_3$–10% DMSO-d_6) δ: 3.71 (3H, s), 5.99 (1H, d, *J*=2.7 Hz), 6.03 (2H, d, *J*=2.2 Hz), 6.03 (1H, d, *J*=2.7 Hz), 6.25 (1H, s).

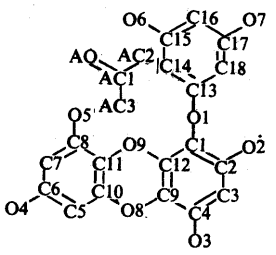
3',4,5'-Trimethoxyeckol (1k), 2,3',5'-Trimethoxyeckol (1l), and 3',5',7-Trimethoxyeckol (1m) A solution (0.79 ml, 0.65 mmol) of 0.84 N BBr_3 in CH_2Cl_2 was added over 2 h to a solution of **1b** (100 mg, 0.219 mmol) in CH_2Cl_2 (4 ml) at −25 °C. The reaction was terminated by the addition of water and then extracted with EtOAc. The EtOAc solution was washed with brine, and dried over $MgSO_4$. The solvent was removed *in vacuo* to give an oil (60 mg), which was subjected to HPLC [sol.: H_2O–MeOH (1 : 1), 3 ml/min, detection by UV 254 nm] and the peaks corresponding to the retention times of 23, 25, and 28 min were collected to yield **1k** (30 mg), **1m** (5 mg), and **1l** (45 mg) as colorless powders. **1k**: mp 104—105 °C. UV λ_{max}^{MeOH} nm (ε): 233 (37000), 290 (3100). EI-MS *m/z* (rel. int.): 414 (M^+, 100), 274 (10), 207 (10). ^{1}H-NMR ($CDCl_3$) δ: 3.75 (6H, s), 3.88 (3H, s), 6.04 (1H, d, *J*=2.7 Hz), 6.08 (1H, d, *J*=2.7 Hz), 6.17 (2H, d, *J*=2.1 Hz), 6.20 (1H, t, *J*=2.1 Hz), 6.34 (1H, s). **1l**: mp 100—101 °C. EI-MS *m/z* (rel. int.): 414 (M^+, 100), 274 (30), 259 (20), 151 (25). ^{1}H-NMR ($CDCl_3$) δ: 3.75 (6H, s), 3.78 (3H, s), 6.02 (1H, d, *J*=2.7 Hz), 6.08 (1H, d, *J*=2.7 Hz), 6.13 (2H, d, *J*=2.1 Hz), 6.15 (1H, t, *J*=2.1 Hz), 6.32 (1H, s). **1m**: mp 100—102 °C. UV λ_{max}^{MeOH} nm (ε): 233 (34600), 292 (5300). EI-MS *m/z* (rel. int.): 414 (M^+, 100), 260 (15). ^{1}H-NMR ($CDCl_3$) δ: 3.70 (3H, s), 3.75 (6H, d, *J*=2.1 Hz), 6.03 (1H, d, *J*=2.7 Hz), 6.07 (1H, d, *J*=2.7 Hz), 6.17 (2H, d, *J*=2.1 Hz), 6.20 (1H, t, *J*=2.1 Hz), 6.34 (1H, s).

Bromination of 3,4-Dimethoxyphenol Bromine (320 mg, 2 mmol) was added dropwise to a solution of dry toluene (10 ml) and *tert*-butylamine (294 mg) at −25 °C, and then the solution was cooled down to −70 °C. Next, a solution of 3,5-dimethoxyphenol (308 mg, 2 mmol) in CH_2Cl_2 (2 ml) was added dropwise at −70 °C. After the completion of the addition the reaction mixture was allowed to stir at room temperature over a period of 2 h and then stirring was continued at room temperature overnight. EtOAc and 2 N HCl were added successively. The organic layer was washed with water, and dried over $MgSO_4$. The removal of the solvent afforded an oil, which was chromatographed on silica gel (10 g) with EtOAc–benzene (1 : 9) to yield 2,6-dibromo-3,5-dimethoxyphenol (300 mg) as prisms from benzene, mp 155—156 °C. IR ν_{max}^{KBr} cm^{-1}: 3480, 1568, 1455, 1410, 1340, 1245. EI-MS *m/z* (rel. int.): 314 (55), 312 (100), 310 (56). ^{1}H-NMR (90 MHz, $CDCl_3$) δ: 3.80 (6H, s), 5.93 (1H, OH), 6.07 (1H, s), and 2-bromo-3,5-dimethoxyphenol (**4**) (110 mg) as prisms (from benzene–MeOH), mp 158—159 °C. IR ν_{max}^{KBr} cm^{-1}: 3380, 1585, 1450, 1430. EI-MS *m/z* (rel. int.): 234 (100), 232 (100), 189, 120. ^{1}H-NMR (90 MHz, $CDCl_3$) δ: 3.66 (3H, s), 3.72 (3H, s), 5.60 (1H, OH), 6.00 (1H, d, *J*=1.8 Hz), 6.17 (1H, d, *J*=1.8 Hz).

Dibenzo-1,4-dioxin-1,3,6,8-tetraol (5) A mixture of 2-bromo-3,5-dimethoxyphenol (**4**) (980 mg), K_2CO_3 (660 mg), cuprous oxide (1.4 g), and dry HMPA (20 ml) was stirred at 180 °C for 2 d. After being cooling at 0 °C, the reaction mixture was acidified with 2 N HCl and extracted with ether. The extract was dried over $MgSO_4$. Evaporation of the solvent left an oil (650 mg), which was chromatographed on silica gel (30 g) with EtOAc–benzene (1 : 9) to give a dimer (40 mg) (*m/z* 304). The obtained

TABLE IV. Atomic Coordinates ($\times 10^{-4}$) for Non-hydrogen Atoms of **1** with Their e.s.d.'s in Parentheses

Atom	x	y	z
O1	4276 (4)	488 (3)	3452 (2)
O2	7060 (4)	3296 (3)	3931 (2)
O3	6689 (3)	4182 (3)	672 (2)
O4	−618 (4)	−2387 (4)	−2017 (2)
O5	−119 (4)	−3279 (3)	1143 (2)
O6	769 (4)	3505 (3)	5635 (2)
O7	1538 (4)	−772 (4)	6542 (2)
O8	3902 (4)	1482 (4)	227 (2)
O9	2609 (4)	−416 (3)	1680 (2)
C1	4824 (5)	1501 (5)	2804 (3)
C2	6279 (5)	2903 (5)	3025 (3)
C3	6905 (5)	3843 (5)	2329 (3)
C4	6098 (5)	3338 (5)	1396 (3)
C5	1709 (5)	−433 (5)	−915 (3)
C6	288 (5)	−1860 (5)	−1133 (3)
C7	−321 (5)	−2829 (5)	−461 (3)
C8	494 (5)	−2327 (5)	465 (3)
C9	4654 (5)	1931 (5)	1169 (3)
C10	2495 (5)	42 (5)	20 (3)
C11	1895 (5)	−879 (5)	713 (3)
C12	4025 (5)	1015 (5)	1873 (3)
C13	3246 (5)	894 (5)	4346 (3)
C14	2559 (5)	2099 (5)	4556 (3)
C15	1500 (5)	2325 (5)	5456 (3)
C16	1186 (5)	1388 (5)	6128 (3)
C17	1895 (5)	177 (5)	5879 (3)
C18	2943 (5)	−105 (5)	4982 (3)
AO	1615 (4)	−3396 (4)	2687 (2)
AC1	3020 (6)	−3574 (5)	2668 (4)
AC2	4437 (6)	−3145 (6)	1843 (4)
AC3	3433 (7)	−4173 (7)	3447 (4)



dimer was dissolved in CH_2Cl_2 (2 ml) and cooled to 0 °C. To the resulting solution, a solution (0.4 ml) of 0.84 N BBr_3 in CH_2Cl_2 was added dropwise at 0 °C. Stirring was continued at 0 °C for 30 min, and then at room temperature for 20 h. The reaction mixture was acidified with 2 N HCl and then extracted with EtOAc. The organic layer was washed with water and brine, and dried over $MgSO_4$. Evaporation of the solvent afforded an oil (26 mg), which was purified by preparative thin layer chromatography (TLC) (2 mm) with MeOH–$CHCl_3$ (3:17) to afford **5** (16 mg) as an amorphous substance. High-resolution MS: Found, m/z 248.0325 (M^+); Calcd $C_{12}H_8O_6$, m/z 248.0321. EI-MS m/z (rel. int.): 248 (M^+, 100), 219 (12), 192 (6), 124 (12), 69 (96). ^{1}H-NMR (DMSO-d_6) δ: 5.78 (1H, d, J = 2.1 Hz), 5.96 (1H, d, J = 2.1 Hz), 9.13 (1H, s, OH), 9.51 (1H, s, OH).

Crystal Data for 1 $C_{18}H_{12}O_9 \cdot C_3H_6O$, triclinic, space group $P\bar{1}$, a = 8.277(4), b = 9.281(5), c = 13.646(9) Å, α = 108.41(5), β = 80.91(5), γ = 112.13(4)°, Z = 2, D_x = 1.55 g/cm^3 and μ (MoK_α) = 1.4 cm^{-1}.

X-Ray Analysis of 1 A single crystal with approximate dimensions of 0.2 × 0.2 × 0.1 mm was chosen for the X-ray study from among crystals grown in acetone–water. The diffraction intensities were measured on a four-circle diffractometer (Syntex R3) using graphite-monochromated MoK_α radiation. The intensities of 1544 peaks were collected as being above the 1.96σ (I) level out of 24022 within the 2θ angle range of 3° through 45°. The structure was solved by the direct method using MULTAN and refined by the block-diagonal least-squares method. The final R value was 0.052. Atomic coordinates and bond distances are given in Tables IV and V, respectively.

Anti-α_2-macroglobulin Activity α_2-Macroglobulin (α-M) was preincubated with a test substance at 37 °C for 20 min and then residual activity of α-M was determined using plasmin (0.6 unit) or trypsin (5 μg) as a protease by the caseinolytic method.[7]

TABLE V. Bond Distances (Å) for Non-hydrogen Atoms of **1** with Their e.s.d.'s in Parentheses

Bond	Distance	Bond	Distance
O1–C1	1.396 (5)	O1–C13	1.407 (5)
O2–C2	1.367 (5)	O3–C4	1.369 (5)
O4–C6	1.375 (5)	O5–C8	1.387 (5)
O6–C15	1.382 (5)	O7–C17	1.380 (5)
O8–C9	1.391 (5)	O8–C10	1.384 (5)
O9–C11	1.404 (5)	O9–C12	1.382 (5)
C1–C2	1.386 (6)	C1–C12	1.390 (6)
C2–C3	1.400 (6)	C3–C4	1.394 (6)
C4–C9	1.384 (6)	C5–C6	1.384 (6)
C5–C10	1.392 (6)	C6–C7	1.392 (6)
C7–C8	1.387 (5)	C8–C11	1.389 (6)
C9–C12	1.393 (6)	C10–C11	1.384 (6)
C13–C14	1.371 (6)	C13–C18	1.393 (6)
C14–C15	1.399 (6)	C15–C16	1.386 (6)
C16–C17	1.384 (6)		
C17–C18	1.397 (6)	AO–AC1	1.230 (6)
AC1–AC2	1.522 (7)	AC1–AC3	1.479 (7)

Anti-α_2-plasmin Inhibitor Activity α_2-Plasmin inhibitor (α-PI) (3 μg) was preincubated with a test substance at 37 °C for 20 min and then 0.05 unit of plasmin in 0.1 ml of 0.1 M sodium phosphate buffer, pH 7.4, containing 25% glycerin (v/v) was added. The mixture was incubated at 37 °C for 30 s, and then 0.1 ml of 3 mM S-2251 was added. After incubation at 37 °C for 30 min, the reaction was terminated by the addition of 0.1 ml of 50% acetic acid and the absorbance of the reaction mixture at 405 nm was determined. The percentage inhibition was calculated as follows; $[(a-b)/(c-b)] \times 100$, where a is the absorbance with α-PI and test substance, b with α-PI but without test substance, and c without α-PI and test substance.

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