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# A New *in vitro* Tissue Factor Inhibitory Triterpene from the Fruits of *Chaenomeles sinensis*



**Abstract**

Tissue factor (TF, tissue thromboplastin) accelerates the blood clotting, activating both the intrinsic and the extrinsic pathways to serve as a cofactor. In order to isolate TF inhibitors from the fruits of *Chaenomeles sinensis*, an activity-guided purification was carried out to yield four triterpenoid compounds. One compound was new and its structure was elucidated as 28-*O*-$\beta$-D-glucopyranosyl-2$\alpha$,3$\beta$-dihydroxyolean-12-ene-24,28-dioic acid (**2**), named chaenomeloside A by means of spectral analysis and chemical conversion. Other compounds were trachelosperoside A-1 (**1**), oleanolic acid (**3**) and ursolic acid (**4**). Compound **2** and its aglycone **2a**, named chaenomelogenin A inhibited by 50% the TF activity at concentrations of 0.036 and 0.028 mM/unit of TF, respectively. Compound **1** isolated for the first time from this plant as well as **3** and **4** were inactive.

**Key words**
*Chaenomeles sinensis* · Rosaceae · 28-*O*-$\beta$-D-glucopyranosyl-2$\alpha$,3$\beta$-dihydroxyolean-12-ene-24,28-dioic acid · chaenomeloside A · chaenomelogenin A · trachelosperoside A-1 · tissue factor inhibitor

## Introduction

TF is a cell surface receptor of FVII (coagulation factor VII) and is the principal initiator of the vertebrate coagulation cascade [1]. Vascular damage exposes blood to cells expressing TF, which participates to form a TF/FVII(a)/phospholipid/$Ca^{2+}$ complex. The complex mediates the activation of both the intrinsic and the extrinsic pathways [2]. This critical position of the TF/FVII(a) complex within the blood coagulation cascade makes it an attractive target for anti-thrombotic drug discovery [3], so the search for TF inhibitors from natural products has a profound significance. We have screened various plants belonging to the Rosaceae for their inhibition on TF by measuring the prothrombin time, and the fruits of *C. sinensis* were found to have a strong inhibitory activity on TF. This report describes the isolation and characterization of a TF inhibitor from the fruits of this plant.

The fruits of *Chaenomeles sinensis* (Thunb.) Koehne (Rosaceae) are used in traditional medicine for the treatment of cough, common cold, pain, diarrhea [4], [5] in Korea, Japan and China. It is known that the fruits contain oleanolic acid, ursolic acid [6], chaenoside A and B [7], maslinic acid, tormentic acid, euscaphic acid [8], 2-hydroxynaringenin-7-*O*-$\beta$-D-glucoside [9], proanthocyanidin [10] and tannin [11], and have an inhibitory effect on tyrosinase [12] as well as an anti-influenza A virus activity [9].

## Materials and Methods

### Materials

The dried fruits of *C. sinensis* were purchased from a Kyungdong market in Seoul, Korea and verified by Emeritus Prof. H. J. Chi, Seoul National University, Korea. A voucher specimen (NPRI 010201) was deposited at the Herbarium of Natural Products Research Institute, Seoul National University, Korea.

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**Animals**

The rats (Sprague-Dawley, 250 ± 20 g) were bred at the Animal Station of Natural Products Research Institute, Seoul National University. They were fed with a commercial solid food (Samyang Yuji Co. Ltd., Seoul) and tap water and were housed at 23 ± 0.5 °C and 60% humidity in a 12 hours light-dark cycle in accordance with the Guide for the Care and Use of Laboratory Animals by Seoul National University.

**General experimental procedures**

Melting point was determined on a Büchi B-540 apparatus and was uncorrected. UV spectra were recorded on a Hitachi U-3210 UV-VIS spectrometer. IR spectra were obtained on a Jasco FT/IR-5300 spectrometer. FAB-MS were measured on a Jeol JMS-SX 102A mass spectrometer and EI-MS on a Hewlett Packard model 5989B GC/MS spectrometer. NMR spectra were recorded on a Varian Gemini-2000 spectrometer or on a Bruker Advance 400 spectrometer operating at 300 or 400 MHz for $^1$H and 75 or 100 MHz for $^{13}$C-NMR. Optical rotations were determined on a Jasco P-1020 polarimeter. Centrifugations were taken at Sorvall RT 6000 centrifuge and Sorvall OTD 65B ultracentrifuge, rotor T 865. GC analysis was carried out on Hewlett Packard 5890 Series II, HP-5 (30 m × 0.32 mm × 0.25 $\mu$m). Silica gel (63 – 200 $\mu$m, Merck KGaA), Sephadex LH-20 (25 – 100 $\mu$m, Sigma Fluka) and LiChroprep RP-18 (40 – 63 $\mu$m, Merck KGaA) were used for open chromatography. TLC was performed on silica gel 60 $F_{254}$ (Merck) and RP-18 $F_{254s}$ (Merck).

**Determination of tissue factor activity by single-stage clotting assay**

A microsomal fraction of rat lung tissue was used as a tissue factor source [13]. A TF stock solution from 5 g of rat lung taken from 4 rats (250 ± 10 g) contained 0.539 mg protein/0.1 mL, when protein concentration was determined according to the Lowry's method [14] using bovine serum albumin as a standard protein. Dilution of the stock solution by 100 – 200 times was used for assay, which gave 5 to 10 units of TF per 100 $\mu$L. Prothrombin time was measured to determine the TF activity by the single-stage clotting assay, using citrated plasma from rats [13], [15].

**Extraction and isolation**

Dried pieces of the fruits of *C. sinensis* (20 kg) were three times extracted with MeOH (54 L). The methanolic extract (4.6 kg) was successively partitioned between *n*-hexane (8.4 L), EtOAc (8.4 L), *n*-BuOH (8.4 L) and $H_2O$ (7 L) to afford 140, 350, 973 and 2960 g of residues, respectively. The EtOAc (350 g) fraction was divided into two parts according to its solubility in 50% MeOH (1200 mL); 50% MeOH soluble (79 g) and insoluble (223 g) parts. Repetitive chromatography of the former part (79 g) over Sephadex LH-20 (500 g) eluting with MeOH-$H_2O$ (1 : 1) (14,000 mL) afforded an active fraction A (19 g) (3800 – 8300 mL) and an inactive fraction B (38 g, 8500 – 12,000 mL). The active fraction was subjected to column chromatography on silica gel (600 g) eluting with $CHCl_3$-MeOH-$H_2O$ (5 : 1 : 0.1, 3 : 1 : 0.1, each 6000 mL) to give an active sub-fraction $A_1$ (6600 – 9000 mL, 1.9 g), which by further chromatography on reverse phase C-18 (350 mL volume; MeOH-$H_2O$, 1 : 1) yielded compounds **1** (705 – 900 mL, 160 mg) and **2** (1140 – 1190 mL, 180 mg). Column chromatography of an aliquot (20 g) of the 50% MeOH insoluble part (223 g) of the EtOAc fraction on silica gel (600 g) eluting with petroleum ether-EtOAc (9 : 1) yielded oleanolic acid **3** (2300 – 3300 mL, 100 mg) and ursolic acid **4** (3600 – 5200 mL, 200 mg).

*Alkaline hydrolysis and methylation* of compounds **1** and **2** were performed by standard procedures. IR, MS and NMR allowed identification of aglycones **1a** and **2a**, and dimethyl esters **1b** and **2b**.

*Sugar moiety of* **2** *and the absolute configuration* were determined to be D-glucose according to the Hara's method [16].

*Trachelosperoside A-1* (**1**) [17]: white powder, $[\alpha]_D^{13}$: + 7.5 (*c* 0.1, MeOH).

*Trachelosperogenin A* (**1a**) [17]: white powder, $[\alpha]_D^{22}$: + 25.0 (*c* 0.1, $CHCl_3$-MeOH, 4 : 1).

*Trachelosperogenin A dimethyl ester* (**1b**) [17], [18]: white powder, $[\alpha]_D^{19}$: + 17.1 (*c* 0.1, $CHCl_3$).

*28-O-β-D-Glucopyranosyl-2α,3β-dihydroxyolean-12-ene-24,28-dioic acid* (**2**): white powder, m. p. > 300 °C, *Rf* = 0.46 on silica gel ($CHCl_3$-MeOH-$H_2O$, 70 : 30 : 4) and 0.07 on RP-18 $F_{254s}$ (60% MeOH), $[\alpha]_D^{13}$: + 15.5 (*c* 0.1, MeOH), UV (MeOH): $\lambda_{max}$ = end absorption only, IR (KBr): $\nu_{max}$ = 3434, 1720, 1701, 1637, 1458, 1275, 1069 cm$^{-1}$, $^1$H- and $^{13}$C-NMR: see Table **1**, FAB-MS: *m/z* = 687.48 $[M + Na]^+$.

*2α,3β-Dihydroxyolean-12-ene-24,28-dioic acid* (**2a**): white powder, m. p. > 300 °C, $[\alpha]_D^{22}$: + 30.5 (*c* 0.1, $CHCl_3$-MeOH 4 : 1); IR (KBr): $\nu_{max}$ = 3436, 1700, 1630, 1064 cm$^{-1}$, $^1$H-NMR ($CDCl_3$, 300 MHz): $\delta$ = 4.00 (1H, ddd, *J* = 10.4, 9.9, 4.5 Hz, H-2), 2.83 (1H, d, *J* = 9.9 Hz, H-3), 5.21 (1H, brs, H-12), 2.76 (2H, dd, *J* = 14.7, 3.9 Hz, H-18), 1.06 (3H, s, H-23), 0.84 (3H, s, H-25), 0.72 (3H, s, H-26), 1.39 (3H, s, H-27), 0.84 (3H, s, H-29), 0.86 (3H, s, H-30); $^{13}$C-NMR ($CDCl_3$, 75 MHz): $\delta$ = 46.0 (C-1), 67.7 (C-2), 82.7 (C-3), 49.1 (C-4), 56.0 (C-5), 19.6 (C-6), 32.5 (C-7), 38.8 (C-8), 46.2 (C-9), 38.1 (C-10), 22.6 (C-11), 121.8 (C-12), 143.5 (C-13), 41.5 (C-14), 27.2 (C-15), 23.0 (C-16), 46.6 (C-17), 41.0 (C-18), 45.5 (C-19), 30.2 (C-20), 33.5 (C-21), 32.2 (C-22), 25.2 (C-23), 180.4 (C-24), 14.2 (C-25), 16.3 (C-26), 23.7 (C-27), 179.6 (C-28), 32.5 (C-29), 23.2 (C-30), EI-MS: *m/z* = 502 $[M]^+$ (18.9%), 456 (18.9), 248 (100), 203 (40.2).

*2α,3β-Dihydroxyolean-12-ene-24,28-dioic acid dimethyl ester* (**2b**): white powder, $[\alpha]_D^{19}$: + 35.4 (*c* 0.1, $CHCl_3$); $^1$H-NMR ($CDCl_3$, 300 MHz): $\delta$ = 4.02 (1H, ddd, *J* = 11.1, 9.9, 5.1 Hz, H-2), 2.87 (1H, m, H-3), 5.28 (1H, t like, H-12), 2.87 (2H, m, H-18), 1.11 (3H, s, H-23), 0.79 (3H, s, H-25), 0.72 (3H, s, H-26), 1.43 (3H, s, H-27), 0.89 (3H, s, H-29), 0.91 (3H, s, H-30), 3.60 (3H, s, $OCH_3$_24), 3.66 (3H, s, $OCH_3$_28); $^{13}$C-NMR ($CDCl_3$, 75 MHz): $\delta$ = 46.1 (C-1), 68.2 (C-2), 83.5 (C-3), 49.1 (C-4), 56.5 (C-5), 20.1 (C-6), 32.7 (C-7), 39.2 (C-8), 46.7 (C-9), 38.3 (C-10), 23.0 (C-11), 122.2 (C-12), 143.6 (C-13), 41.7 (C-14), 27.6 (C-15), 23.6 (C-16), 46.9 (C-17), 41.3 (C-18), 45.7 (C-19), 30.7 (C-20), 33.8 (C-21), 32.3 (C-22), 25.7 (C-23), 178.5 (C-24), 14.5 (C-25), 16.7 (C-26), 23.8 (C-27), 178.2 (C-28), 33.1 (C-29), 23.6 (C-30), 51.5 ($O\underline{C}H_3$_24), 51.5 ($O\underline{C}H_3$_28), EI-MS: *m/z* = 530 $[M]^+$ (1.7%), 470 (1.7), 262 (34.7), 203 (100).

Table **1** $^{13}C$- and $^{1}H$-NMR spectra of compound **2** from the fruits of *C. sinensis*

| *Carbon no.* | *$^{13}C$* | *$^{1}H$, mult (J)* |
|---|---|---|
| 1 | 48.0 | |
| 2 | 68.2 | 4.73 (ddd, *J* = 9.9, 9.3, 4.5 Hz) |
| 3 | 83.9 | 3.39 (d, *J* = 9.3 Hz) |
| 4 | 49.7 | |
| 5 | 56.5 | |
| 6 | 20.6 | |
| 7 | 33.1 | |
| 8 | 39.6 | |
| 9 | 47.3 | |
| 10 | 38.7 | |
| 11 | 23.1 | |
| 12 | 122.6 | 5.42 (t like) |
| 13 | 143.8 | |
| 14 | 42.0 | |
| 15 | 27.9 | |
| 16 | 23.8 | |
| 17 | 46.7 | |
| 18 | 41.5 | 3.18 (dd, *J* = 14.7, 4.5 Hz) |
| 19 | 45.8 | |
| 20 | 30.5 | |
| 21 | 33.7 | |
| 22 | 32.2 | |
| 23 | 25.7 | 1.22 (s) |
| 24 | 180.3 | |
| 25 | 15.0 | 0.89 (s) |
| 26 | 17.2 | 0.86 (s) |
| 27 | 24.8 | 1.76 (s) |
| 28 | 176.1 | |
| 29 | 32.8 | 1.16 (s) |
| 30 | 23.4 | 1.21 (d, *J* = 6.3 Hz) |
| G-1 | 95.5 | 6.29 (d, *J* = 7.8 Hz) |
| 2 | 73.8 | 3.99 – 4.46 (m) |
| 3 | 78.6 | 3.99 – 4.46 (m) |
| 4 | 70.8 | 3.99 – 4.46 (m) |
| 5 | 79.1 | 3.99 – 4.46 (m) |
| 6 | 61.9 | 3.99 – 4.46 (m) |

* In pyridine-$d_5$, G: $\beta$-D-glucose; $^{1}H$- and $^{13}C$-NMR at 400 and 100 MHz, respectively. The assignment was based upon DEPT (75 MHz), COSY (300 MHz), HMQC (400 MHz) and HMBC (400 MHz) experiments.

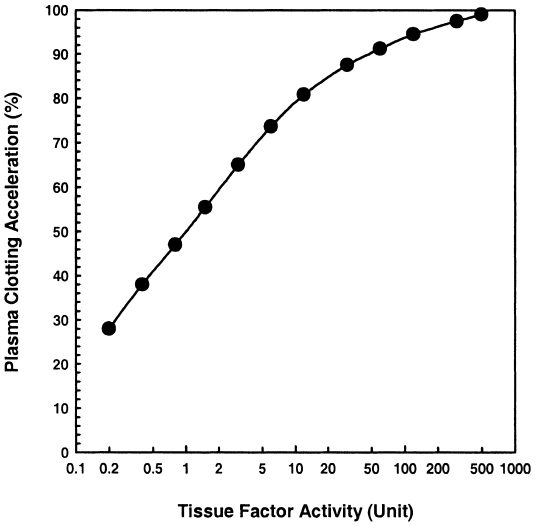


Fig. **1** A standard curve for tissue factor activity of rat lung tissues. The curve was made from the measurements of the prothrombin time assayed on the lung tissues from 25 rats over several times against the pooled plasma from 9 rats. The TF activity was assessed by the single-stage clotting assay as follows: in a plastic test tube prewarmed on a 37 °C water bath, 100 $\mu$L of plasma, 100 $\mu$L of TF solution diluted to the proper concentration with saline and 100 $\mu$L of 25 mM $CaCl_2$ were taken, and prothrombin time was measured after the addition of $CaCl_2$. It was considered as 100% activity when the prothrombin time with TF was 18 sec, and the plasma recalcification time without TF was taken as 0% activity. The activity of TF that gave 50% acceleration of the prothrombin time on the standard curve was arbitrarily defined as one unit of TF.

*Oleanolic acid* (**3**) [6]: white powder, m. p. 310 – 312 °C, $[\alpha]_D^{21}$: + 83 (*c* 0.6, $CHCl_3$); all other data were as in [6].

*Ursolic acid* (**4**) [6]: white powder from EtOH, m. p. 283 – 285 °C, $[\alpha]_D^{21}$: + 66 (*c* 1.0, EtOH); all other data were as in [6].

## Results and Discussion

A variety of assay for tissue factor (TF) has been developed [15]. We have found the single-stage clotting assay of total procoagulant activity in rat tissues to be simple and reproducible [13], [15]. Fig. **1** shows a typical standard curve for tissue factor activity of rat lung tissue determined by the single-stage clotting as-

Table **2** Tissue factor inhibitory activities of various solvent fractions from the fruits of *C. sinensis*

| *Fractions* | *Amount (g)* | *$IC_{50}$/TF unit* (μg/unit)* | *Total inhibitory activity ($\times 10^7$ units**)* | *Specific inhibitory activity ($\times 10^5$ units/g)* |
|---|---|---|---|---|
| *n*-Hexane | 140 | 10.0 | 1.4 | 1.0 |
| EtOAc | 350 | 0.6 | 58.3 | 16.7 |
| *n*-BuOH | 973 | 5.3 | 18.4 | 1.9 |
| $H_2O$ | 2960 | 2.3 | 128.7 | 4.3 |

* The activity of TF that accelerated by 50% the prothrombin time was arbitrarily defined as one unit of TF, when the prothrombin time was determined by the single-stage clotting assay (see the legend of Fig. **1**).

** The amount ($\mu$g) of sample that inhibited by 50% TF activity was defined as one inhibitory unit of TF inhibitor.

say method. Solvent fractionation of the MeOH extract of the fruits of *C. sinensis* revealed that the EtOAc soluble fraction had the strongest inhibitory activity on TF (Table **2**). Activity-guided isolation of the EtOAc fraction yielded compound **2** as a potent TF inhibitor and compounds **1**, **3** and **4** that were inactive in the assay system used.

Compound **1** was obtained as white powder. The FAB-MS of **1** showed a quasi-molecular ion at $m/z$ = 698.2 $[M + NH_4]^+$, which corresponds to a molecular formula of $C_{36}H_{56}O_{12}$. In comparisons of the NMR spectra of **1** with those of trachelosperoside A-1, the 28-*O*-$\beta$-D-glucopyranoside of 2$\alpha$,3$\beta$,19$\alpha$-trihydroxyurs-12-ene-24,28-dioic acid [17], the data of both the compounds were in good agreement.

The FAB-MS of **2** showed a quasi-molecular ion at $m/z$ = 687.48 $[M + Na]^+$, which corresponds to a molecular formula of $C_{36}H_{56}O_{11}$. The presence of an ester glucoside was confirmed by the observation of an anomeric proton at $\delta$ = 6.29 ($J$ = 7.8 Hz) and of an anomeric carbon at $\delta$ = 95.5 (Table **1**). Compound **2** yielded D-glucose on acid hydrolysis and a genin **2a** ($m/z$ = 502, $C_{30}H_{46}O_6$) on alkaline hydrolysis. The $^1$H-NMR spectrum of the genin **2a** exhibited signals due to two protons on vicinal carbons at $\delta$ = 4.00 ($J$ = 10.4, 9.9, 4.5 Hz) and 2.83 ($J$ = 9.9 Hz), disclosing the presence of 2$\alpha$,3$\beta$-dihydroxy groups. Compound **2a** could be deduced as an olean-12-enedioic acid from the presence of two olefinic carbon signals at $\delta$ = 121.8 and 143.5, and of two carboxyl group signals at $\delta$ = 179.6 and 180.4 in the $^{13}$C-NMR spectrum [18], [19]. Methylation of **2a** afforded a dimethyl ester (**2b**) ($m/z$ = 530, $C_{32}H_{50}O_6$) upon treatment with diazomethane. The EI-MS of **2b** showed the characteristic fragment RDA ion peaks at $m/z$ = 262 and 203 due to rings D and E, indicating the absence of 19-OH. In the $^{13}$C-NMR spectrum of **2**, the signals ascribable to the carbons of rings A and B showed the similar chemical shifts to those of the 28-glucopyranosyl ester of 2$\alpha$,3$\beta$,19$\alpha$-trihydroxy-12-oleanen-24,28-dioic acid [19], indicating that one carboxyl group was located on the 24-position. In an HMBC experiment of **2**, a carbon having a signal at $\delta$ = 176.1 (C-28) gave a cross-peak to the anomeric proton at $\delta$ = 6.29 and a carbon at $\delta$ = 180.3 (C-24) had a cross-peak to the 3$\alpha$-proton at $\delta$ = 3.39, indicating that the glucopyranosyl group is located at the position 28 in the aglycone. On the basis of these data, **2** was determined to be the 28-*O*-$\beta$-D-glucopyranoside of 2$\alpha$,3$\beta$-dihydroxyolean-12-ene-24,28-dioic acid (Chart **1**). Compound **2** and the aglycone **2a** were named chaenomeloside A and chaenomelogenin A, respectively.

Compounds **3** and **4** were identified as oleanolic acid and ursolic acid, respectively, in comparison of optical rotation, IR, $^1$H- and $^{13}$C-NMR data with those already isolated from the fruits of *C. sinensis* by other researchers [6].

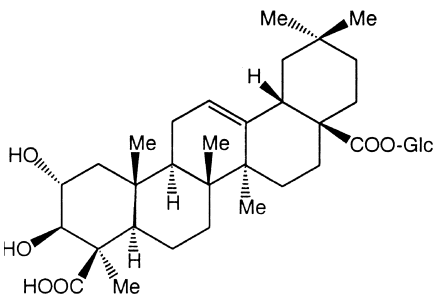


Chart **1** Structure of compound **2**.

**Prothrombin time elongation by 2 and 2a**

Fig. **2** shows that **2** and **2a** did not change the plasma recalcification time in the absence of TF, but elongated the prothrombin time in the presence of TF with the manner of dose dependence. The results suggested that **2** and **2a** did not affect the intrinsic coagulation factors, but TF, the initiator of the extrinsic pathway of blood coagulation did. The inhibition percentages of **2** and **2a** on TF activity as shown in Fig. **3** were obtained by calculation of the decreased extent of TF activity in units from the data under the presence of 9.8 units of TF as shown in Fig. **2**. Other compounds such as **1**, **1a**, **1b**, **3** and **4** did not change the plasma clotting time in the absence and the presence of TF (data not shown).

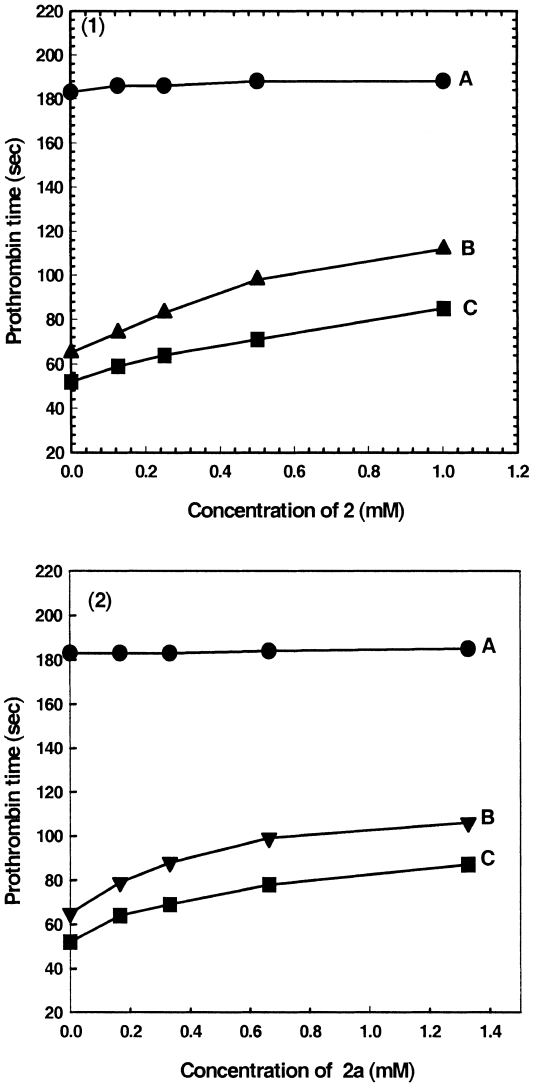


Fig. **2** Prothrombin time elongation by compound **2** (1) and its aglycone **2a** (2) in the presence of rat lung tissue factor. A mixture of 360 $\mu$L of TF solution and 40 $\mu$L of sample solution in 0.1 M Tris buffer (pH 7.4) was preincubated at 37 °C for 20 min, and an aliquot (100 $\mu$L) of the mixture was added to 100 $\mu$L of plasma prewarmed at 37 °C, and the prothrombin time was measured after the addition of 100 $\mu$L of 25 mM $CaCl_2$. Curve **A**: the plasma recalcification time without the addition of TF solution to plasma (control). Curves **B** and **C**: the prothrombin time with the addition of 4.9 and 9.8 units of TF solution to plasma, respectively.

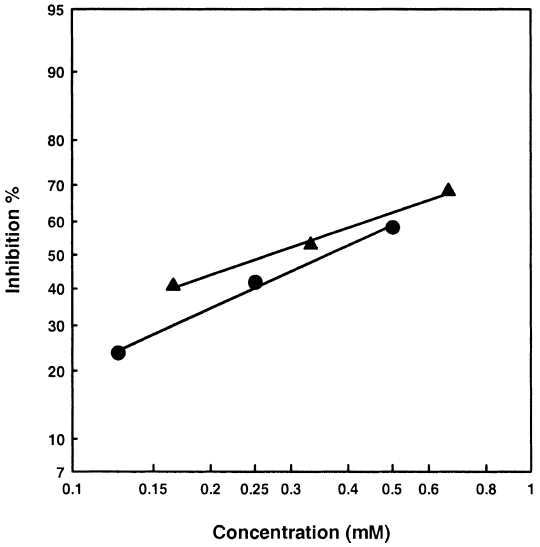


Fig. **3** The inhibition percentage of compound **2** and its aglycone **2a** on tissue factor activity. The percentage was calculated by decrease extent of TF activity in units obtained from the data under the presence of 9.8 units of TF as shown in Fig. **2**. **2** (●) and **2a** (▲). The $IC_{50}$ value of TF inhibitor was obtained from a logit-log graph (a $\%B/B_o$ – log graph) on relationship between the inhibition percentage and the concentration of the inhibitor in log scale. A positive control was not tested because of unavailability.

### TF inhibitory activity and SAR analysis

Among the compounds isolated, only two compounds **2** and **2a** inhibited the TF activity. The aglycone **2a** ($IC_{50}$ = 0.028 mM/unit of TF) was found to be more potent than its glycoside **2** ($IC_{50}$ = 0.036 mM/unit of TF) as shown in Fig. **3**. The dimethyl ester derivative **2b** did not show the inhibitory activity. Moreover, oleanolic acid **3** and ursolic acid **4** that contain one carboxyl group, and **1** and **1a** that contain the 19$\alpha$-hydroxy group, were inactive. These results indicated that the presence of two free carboxyl groups of **2a** could play an important role in exerting the inhibitory activity on TF. In addition, the 19$\alpha$-hydroxy group in the E-ring of compounds **1** and **1a** caused profound changes in activity, suggesting that the presence of a hydrophilic group in that position might not be required for activity. This discovery could provide a possibility to find new drug candidates from natural product resources for regulating the blood coagulation cascade. It may be possible in a near future after further studies to design and synthesize more effective TF inhibitors based on the compounds isolated from *C. sinensis*.

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