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Inhibitory effects of urinary metabolites on platelet aggregation after orally administering Shimotsu-To, a traditional Chinese medicine, to rats

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Abstract

Shimotsu-To, which consists of four herbal extracts, has been used clinically for improving abnormal blood coagulation, fibrinolysis, atherosclerosis and chronic inflammation in Japan and China. We have investigated the pharmacological relationship between the effects and chemical components of Shimotsu-To after oral administration to rats. The urinary constituents were separated and identified by three dimensional (3D-) HPLC equipped with a photodiode array detector as a new tool and the chemical structures were determined by spectroscopic methods to be *trans*-ferulic acid-3-*O*-sulfate (**1**), vanillic acid (**2**), *m*-hydroxyphenylpropionic acid (**3**), *trans*-ferulic acid (**4**) and *cis*-ferulic acid (**5**). Of these compounds, **2**–**5** strongly inhibited platelet aggregation induced by ADP and arachidonic acid. Compound **1**, the sulfate conjugate of **4**, did not show any inhibitory effect, which suggested that the inhibitory effect on platelet aggregation was inactivated by sulfate conjugation. These results indicated that compounds **2**–**5** partly contributed to the anti-Oketsu effect of Shimotsu-To through the inhibition of platelet aggregation.

Introduction

Shimotsu-To is a traditional Chinese medicine consisting of four herbal extracts, cnidium rhizome, peony root, angelica root and rehmannia root, in a preparation of the same dry weight. It has been used clinically for improving the "Oketsu" (blood stagnant) state in traditional Chinese medicine. The "Oketsu" syndrome is recognized as a stagnancy of the blood, including blood coagulation, fibrinolysis, atherosclerosis and chronic inflammation (Yakazu 1975; Terasawa et al 1983). Accordingly, the effects of Shimotsu-To on these syndromes have been investigated from the aspect of blood diseases (Terasawa et al 1983; Ishida et al 1987; Kobayashi et al 1992; Kojima et al 1994), but not of platelet aggregation. Furthermore, the active components contained in Shimotsu-To have not been examined in detail and there has been no detailed investigation as to what kind and what amounts of chemical components are absorbed into the body and of their pharmacological effects. Therefore, we have investigated the pharmacological relationship between the effects and chemical components of Shimotsu-To in rats with the aim of determining the active compounds.

We report the chemical structures of the urinary constituents of orally administered Shimotsu-To to rats, and their inhibitory effects on platelet aggregation induced by ADP and arachidonic acid (AA).

Materials and Methods

Apparatus

IR spectra were measured with a Perkin Elmer FT-IR 1725X spectrometer. NMR spectra were recorded on a JEOL JNM-EX 270 with tetramethylsilane as the internal standard and chemical shifts are given as δ values. Mass spectra (MS) were measured

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with a JEOL DX-303 mass spectrometer. The HPLC system consisted of a CCPM pump, CO-8010 column oven (Tosoh, Tokyo, Japan) and model M991J photodiode array detector (Waters Millipore, Milford, MA).

Reagents

trans-Ferulic acid, vanillic acid and *m*-hydroxyphenylpropionic acid were from Tokyo Chemical Industry Co., Ltd (Tokyo, Japan). *cis*-Ferulic acid was prepared from *trans*-ferulic acid by visible light irradiation. *trans*-Ferulic acid-4-*O*-sulfate was synthesized from *trans*-ferulic acid in the usual way using the sulfur trioxide–pyridine complex. All other reagents were of special grade.

Animals

The study was performed according to the Guide for Care and Use of Laboratory Animals at Tohoku Pharmaceutical University. Male Sprague-Dawley rats (Japan SLC Inc.), 6-weeks old, were maintained under a constant temperature ($22 \pm 2\,^{\circ}C$) and humidity ($55 \pm 10\%$), with free access to commercial food pellets (CA-1, Clea Japan, Tokyo) and tap water. The room had a 12-h light/12-h dark cycle. For 18 h before the experiments the rats were deprived of food but had free access to water.

Preparation of extracts of Shimotsu-To, *Cnidium officinale* and *Angelica acutiloba*

To 600 mL of distilled water was added either Shimotsu-To (3.0 g each of rhizome of *C. officinale*, root of *A. acutiloba*, root of *Paeonia lactiflora* and root of *Rehmannia glutinosa*), the rhizome (3.0 g) of *C. officinale*, or the root (3.0 g) of *A. acutiloba*, and boiled until the volume was reduced to 300 mL. Each filtered decoction was freeze-dried and the obtained powder (Shimotsu-To: 4.5 g; *C. officinale*: 0.8 g; *A. acutiloba*: 1.2 g) was stored below $-20\,^{\circ}C$ until use.

Preparation of urine samples

trans-Ferulic acid ($100\,mg\,kg^{-1}$), extract of Shimotsu-To ($3.0\,g\,kg^{-1}$) or *C. officinale* ($3.0\,g\,kg^{-1}$) was orally administered to rats. With the use of a metabolic cage, urine specimens were obtained over 24 h.

Detection of ***1***
Each 1.0 mL of urine was added to 5 mL methanol. The solution was filtered through a 0.45-μm membrane filter and 20 μL of the filtered solution was injected into an HPLC column.

Detection of ***2–5***
Each 1.0 mL of urine described above was transferred to a test tube followed by the addition of 7.0 mL 0.2 M sodium acetate buffer (pH 5.5) and 50 μL β-glucuronidase/arylsulfatase solution (type H-1, Sigma, USA). The incubation mixture was transferred to a separating funnel and extracted three times with ethyl acetate (30 mL). The organic layer was washed with 1 mL water and evaporated to dryness at $40\,^{\circ}C$. The residue was dissolved in 1 mL methanol and a 20-μL sample was injected onto an HPLC column.

HPLC conditions and method validation

A stainless steel column (250×4.6 mm i.d.), packed with reversed-phase TSK gel ODS-120T (5 μm, Tosoh Company Ltd, Tokyo, Japan) was used. The mobile phase was a linear gradient system comprised of 0.05% trifluoroacetic acid (solvent A) and 100% acetonitrile (solvent B).

Detection of ***1***
A/B = 95/5 (0 min) → 90/10 (30 min) → 80/20 (60 min).

Detection of ***2–5***
A/B = 95/5 (0 min) → 80/20 (30 min) → 70/30 (40 min).

The flow rate was $1.0\,mL\,min^{-1}$ at $40\,^{\circ}C$. The detection wavelength of urinary metabolites was set at 200–400 nm. The peak purity of each urinary metabolite was checked by photo diode-array detection. The UV spectra of peaks at three different points (up-slope, top and down-slope) were compared with those of authentic specimens. The interday precision of the method for detection of each urinary metabolite was evaluated using urine samples and was examined with three replicate assays per day. The interday relative standard deviations were within 3.0% (data not shown). The detection limits (signal-to-noise ratio = 3, UV at 220 nm) for **1–5** were 20, 50, 50, 20 and $50\,ng\,mL^{-1}$, respectively.

Isolation of 1–5

For the isolation of **1**, 1.0 g of *trans*-ferulic acid was orally administered in portions to each of the 12 rats at $100\,mg\,kg^{-1}$/day over a period of two weeks and the urine samples were collected using metabolic cages. The combined urine sample (220 mL) was chromatographed on Sephadex LH-20 (0.05 M HCl → H_2O → MeOH) and the H_2O eluate fraction containing **1** was subjected to repeated chromatography on a Sephadex LH-20 (H_2O → MeOH) to afford **1** (8 mg). For isolation of **2–5**, the C. *officinale* extracts suspended in 0.5% CMC-Na solution were orally administered in portions to each of the 12 rats at $3.0\,g\,kg^{-1}$/day over a period of 60 days and urine samples were collected using metabolic cages. The combined urine samples (570 mL) were incubated with β-glucuronidase/arylsulfatase (type H-2, Sigma, USA) in 0.2 M sodium acetate buffer (pH 5.5) for 24 h at $37\,^{\circ}C$. The incubated solution was extracted three times with ethyl acetate. The organic layer was evaporated to dryness at $40\,^{\circ}C$. The residue (400 mg) was dissolved in a small amount of MeOH and chromatographed on Sephadex LH-20 with H_2O and MeOH as the eluant. The fraction containing **3–5** was subjected to repeated chromatography on a Sephadex LH-20 (H_2O → MeOH) to give **3** (4 mg), **4** (3 mg) and **5** (10 mg). The fractions containing **2** were subjected to

preparative HPLC under the following conditions: column, TSKgel ODS-120T (10 μm, 300 × 7.8 mm i.d., Tosoh Company Ltd, Tokyo, Japan); mobile phase, 0.05% trifluoroacetic acid (solvent A) and 100% acetonitrile (solvent B), linear gradient system, A/B = 95/5 (0 min) → 80/20 (80 min) → 70/30 (100 min) → 60/40 (120 min). The flow rate was 2.0 mL min^{-1} at room temperature. The fraction containing **2** was evaporated to dryness at 40 °C in-vacuo to give **2** (8 mg).

1 *(trans-ferulic acid-4-O-sulfate)*
Colourless amorphous powder, mp 228–230 °C. IR (KBr) ν_{max} 1648, 1592, 1056, 984 cm^{-1}. ^{1}H NMR (CD_3OD, 270 MHz) δ: 3.75 (3H, s, —OCH_3), 6.38 (1H, d, J = 15.5 Hz, H-8), 6.98 (1H, dd, J = 8.5, 1.7 Hz, H-6), 7.10 (1H, d, J = 1.7 Hz, H-2), 7.15 (1H, d, J = 15.5 Hz, H-7), 7.41 (1H, d, J = 8.5 Hz, H-5). ^{13}C NMR (CD_3OD, 65 MHz) δ: 55.7 (—OCH_3), 110.7 (C-2), 119.4 (C-6), 120.8 (C-5), 127.3 (C-8), 131.8 (C-1), 137.0 (C-7), 143.0 (C-4), 150.6 (C-3), 171.7 (C-9). Negative FABMS m/z: 295 $(M - H + Na)^-$, 273 $(M-H)^-$, 193 $(M - H - SO_3)^-$.

2 *(vanillic acid)*
^{1}H NMR (CD_3OD, 270 MHz) δ: 3.89 (3H, s, —OCH_3), 8.41 (1H, d, J = 6.8 Hz, H-5), 7.52 (1H, dd, J = 2.0 Hz, H-6), 7.56 (1H, d, J = 2.0 Hz, H-2). EIMS m/z: 168 (M^+).

3 *(m-hydroxyphenyl-propionic acid)*
^{1}H NMR (CD_3OD, 270 MHz) δ: 2.54 (2H, t, J = 7.8 Hz), 2.83 (2H, t, J = 7.8 Hz), 6.59 (1H, dd, J = 7.4, 2.5 Hz), 6.65 (1H, d, J = 2.5 Hz), 6.68 (1H, t, J = 7.4 Hz), 7.07 (1H, t, J = 7.8 Hz). EIMS m/z: 166 (M^+).

4 *(*trans*-ferulic acid)*
^{1}H NMR (CD_3OD, 270 MHz) δ: 3.88 (3H, s, –OCH_3), 6.33 (1H, d, J = 15.8 Hz), 6.75 (1H, d, J = 8.2 Hz), 6.97 (1H, dd, J = 8.2, 2.0 Hz), 7.11 (1H, d, J = 2.0 Hz), 7.31 (1H, d, J = 15.8 Hz). EIMS m/z: 194 (M^+).

5 *(*cis*-ferulic acid)*
^{1}H NMR (CD_3OD, 270 MHz) δ: 3.84 (3H, s, —OCH_3), 5.89 (1H, d, J = 12.7 Hz), 6.23 (1H, dd, J = 12.7, 2.0 Hz), 6.69 (1H, d, J = 8.2 Hz), 6.92 (1H, dd, J = 8.2, 2.0 Hz), 7.44 (1H, d, J = 2.0 Hz). EIMS m/z: 194 (M^+).

Assay of platelet aggregation

Blood was collected from a healthy rat and mixed with 3.8% sodium citrate at the ratio (v/v) of 10:1. The platelet-rich plasma (PRP) was prepared by centrifugation of the blood at 1000 rev min^{-1} for 20 min, and the platelet-poor plasma (PPP) was prepared by centrifugation of the remaining blood residue at 3000 rev min^{-1} for 20 min. The platelet count was adjusted to 250–300 × 10^6 mL^{-1}. The platelet aggregation measurement was carried out using a photometric method with an automated platelet aggregometer analyser (Tokyo Photo Electric Co. Ltd, Tokyo, Japan) and was performed in duplicate with ADP (1 mM) or AA (30 mM) as the aggregating reagents. A 200-μL sample of PRP was incubated with 20 μL of a test solution at 37 °C for 5 min, then 20 μL of an aggregating reagent was added to the reaction mixture. All the test compounds were tested at a fixed dose of 250 and 1000 μg mL^{-1}. The aggregation profile was recorded in terms of the change in PRP light transmittance with constant stirring. The inhibition percent was calculated using the following equation:

$$\% \text{ inhibition} = (1 - \text{sample value}/\text{control value}) \times 100$$

Each value represents the mean ± s.e.m of three experiments.

Statistical analysis

All data are expressed as mean ± s.e.m. The inhibitory data was analysed using a Kruskal-Wallis test. If any statistically significant change was found, post-hoc comparison was performed using the Fisher's LSD multiple range test. Data was deemed significant when $P < 0.05$.

Results

By using 3D-HPLC, one distinct peak, **1** (R_f 13 min) was detected in the enzyme non-treated urine of rats orally administered Shimotsu-To. Otherwise, four peaks, **2** (R_f 17 min), **3** (R_f 26 min), **4** (R_f 28 min) and **5** (R_f 30 min), were detected after pretreating the urine with β-glucuronidase/arylsulfatase. We examined the origin of **1–5** using the HPLC of each herbal extract. As a result, **1–3** were found to be derived from *trans*-ferulic acid (**4**), a major phenolic constituent in *C. officinale* and *A. acutiloba* (Naito et al 1995). Furthermore, it was found that **5** was derived from **4** during preparation of the extract in boiling water (data not shown).

Compounds **1–5** were isolated from urine samples by chromatographic separation on a Sephadex LH-20 column and repeated preparative HPLC followed by determination of these chemical structures.

Enzymatic hydrolysis of **1** with arylsulfatase gave a product that was identified as *trans*-ferulic acid by direct comparison of UV spectrum and R_f values on HPLC with those of an authentic sample. The IR spectrum of **1** showed absorption bands at 1648, 1592 and 984 cm^{-1} due to conjugated carboxyl and *trans* double-bond functions. Intense absorption at 1056 cm^{-1} indicated **1** to have possibly a sulfate-conjugated structure. Negative FABMS of **1** exhibited a molecular ion peak at m/z 273 $(M—H)^-$ along with a fragment ion peak at m/z 193 $(M—H—SO_3)^-$ corresponding to mono-sulfate. A sulfate group is thus shown to be present in **1**. A comparison of the ^{13}C NMR spectrum of **1** with that of *trans*-ferulic acid indicated the C-4 signal of **1** to have shifted 6.0 ppm upfield, accompanied by downfield shifts of C-3 (2.8 ppm) and C-5 (5.4 ppm). These shifts indicated that the sulfate group was attached to C-4. The structure of **1** was concluded to be *trans*-ferulic acid-4-*O*-sulfate. Direct comparison of **1** with an authentic sample synthesized from *trans*-ferulic acid by IR, FABMS and ^{1}H NMR spectra revealed its identity.

Compounds **2–5** were identified as vanillic acid, *m*-hydroxyphenyl-propionic acid, *trans*-ferulic acid and *cis*-ferulic acid, respectively, by direct comparison of their ^{1}H NMR and MS data with authentic samples. The chemical structures of **1–5** are depicted in Figure 1.

The inhibitory activities of compounds **1–5** on platelet aggregation induced by ADP and AA are shown in Figures 2 and 3, respectively. There were significant effects ($P < 0.05$, Kruskal-Wallis test) of test compound treatment on platelet aggregation induced by ADP and AA, with the exception of compound **3** on platelet aggregation by AA. Post-hoc analysis revealed that compounds **2–5** dose-dependently inhibited the ADP-induced platelet aggregation (Figure 2). The inhibitory activities of compounds **2–5** at a concentration of 250 μg mL^{-1} were 20.1 ± 3.5% (**2**), 17.1 ± 1.2% (**3**), 41.4 ± 1.7% (**4**) and

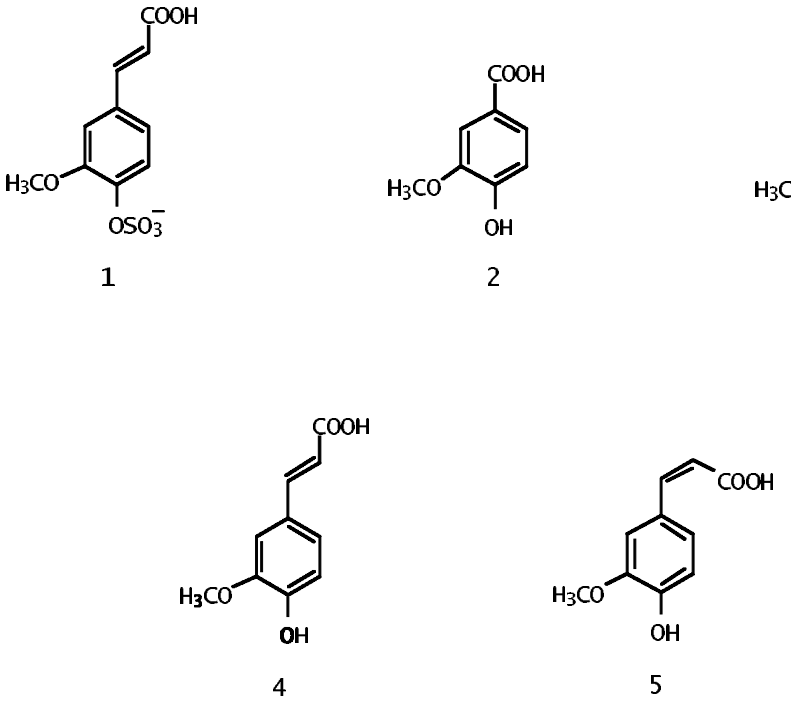

Figure 1 Chemical structures of *trans*-ferulic acid-4-*O*-sulfate (**1**), vanillic acid (**2**), *m*-hydroxyphenyl-propionic acid (**3**), *trans*-ferulic acid (**4**) and *cis*-ferulic acid (**5**).

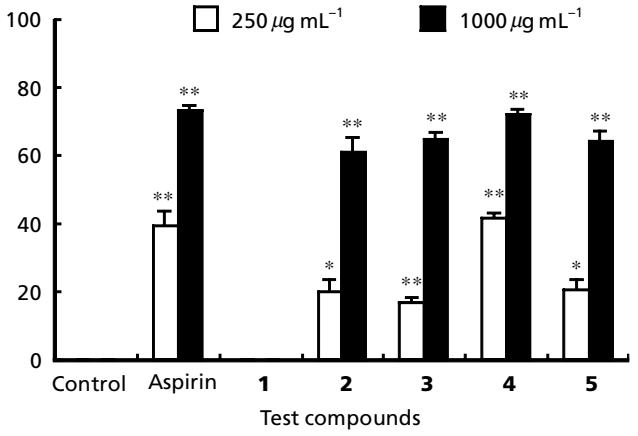

Figure 2 Inhibitory effects of **1–5** and aspirin on ADP-induced platelet aggregation. **1**, *trans*-ferulic acid-4-*O*-sulfate; **2**, vanillic acid; **3**, *m*-hydroxyphenyl-propionic acid; **4**, *trans*-ferulic acid; **5**, *cis*-ferulic acid. Each value is the mean ± s.e.m. of three experiments. *$P < 0.01$, **$P < 0.001$ (Fisher's LSD multiple range test).

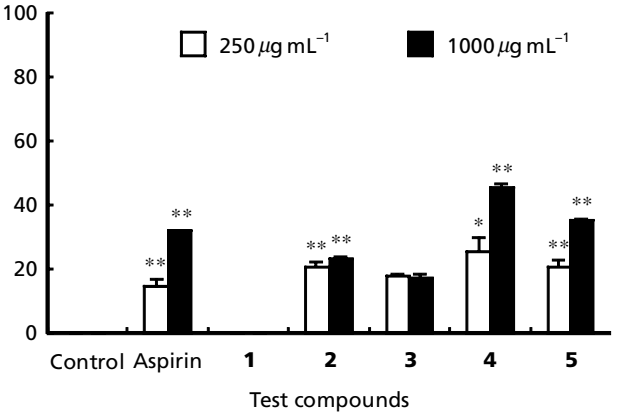

Figure 3 Inhibitory effects of **1–5** and aspirin on arachidonic acid-induced platelet aggregation. **1**, *trans*-ferulic acid-4-*O*-sulfate; **2**, vanillic acid; **3**, *m*-hydroxyphenyl-propionic acid; **4**, *trans*-ferulic acid; **5**, *cis*-ferulic acid. Each value is the mean ± s.e.m. of three experiments. *$P < 0.01$, **$P < 0.001$ (Fisher's LSD multiple range test).

$20.7 \pm 3.0\%$ (**5**), and at a concentration of 1000 $\mu g\,mL^{-1}$ were $60.8 \pm 4.5\%$ (**2**), $65.0 \pm 1.9\%$ (**3**), $72.0 \pm 1.7\%$ (**4**) and $64.0 \pm 3.4\%$ (**5**), respectively. Compound **4** (250 $\mu g mL^{-1}$), as well as the positive control agent, aspirin ($39.6 \pm 3.9\%$), showed an inhibitory effect. Furthermore, compounds **2**–**5** at a concentration of 1000 $\mu g\,mL^{-1}$ showed a potent inhibitory effect as did aspirin ($73.0 \pm 1.7\%$), whereas compound **1**, the sulfate conjugate of **4**, had no effect. Figure 3 shows that the inhibitory activities of compounds **1**–**5** at a concentration of 250 $\mu g\,mL^{-1}$ were 0% (**1**), $20.5 \pm 1.4\%$ (**2**), $17.7 \pm 0.6\%$ (**3**), $25.2 \pm 4.3\%$ (**4**) and $20.5 \pm 2.0\%$ (**5**), and at a concentration of 1000 $\mu g\,mL^{-1}$ were 0% (**1**), $23.5 \pm 0.3\%$ (**2**), $17.5 \pm 0.9\%$ (**3**), $45.3 \pm 1.4\%$ (**4**) and $35.0 \pm 0.6\%$ (**5**). Although compounds **2**–**5** (250 $\mu g\,mL^{-1}$) weakly inhibited the platelet aggregation induced by AA, compounds **4** and **5** at a concentration of 1000 $\mu g\,mL^{-1}$ showed a strong inhibitory effect as well as that of aspirin ($32.0 \pm 2.0\%$).

Discussion

"Oketsu", blood stasis or stagnant state, which is one of the pathological concepts in oriental medicine, has been defined to be caused by a change in blood flow and blood conditions (Terasawa 1983, 1998). This syndrome is related to serial diseases, such as systemic lupus erythematosus, rheumatoid arthritis, cerebrovascular disease and many inflammations. Several studies have been performed to elucidate the mechanism of the "Oketsu" state (Terasawa et al 1986) and the effects of the anti-"Oketsu" traditional Chinese medicine (Tosa et al 1987). Haematological studies of the "Oketsu" syndrome elucidated that thromboxane synthesis was elevated in the platelets of patients with this syndrome, and the anti-"Oketsu" traditional Chinese medicines corrected the condition (Terasawa et al 1985). Reports on the rheological aspects of the blood in the "Oketsu" syndrome have pointed out the elevation of the blood viscosity (Arichi et al 1981). In Japan and China, various traditional herbal medicine composite formulations are commonly used in clinical practice. The study of the drug's pharmacokinetics would help to understand the mechanism of its biological activity. Also, it is important to improve the efficacy and safety of the drug. In addition, a pharmacokinetic study would provide a scientific explanation for the efficacy of the herbal medicine composite formulations that have been used based only on empirical findings.

A number of pharmacokinetic studies of plant phenolic compounds, mainly flavonoids, have been reported because of their numerous physiological functions. Recently, it was reported that orally administered curcuminoids were absorbed from the alimentary tract of rats and entered the general blood circulation where they were present largely as glucuronide and glucuronide/sulfate conjugates (Asai & Miyazawa 2000).

In this study, we have determined the urinary components of Shimotsu-To after oral administration to rats, and which components inhibited platelet aggregation induced by ADP and AA. All urinary components were phenolic compounds and were mainly excreted in the urine as the sulfate and/or glucuronic acid conjugation form. The results suggested that the inhibitory effect on platelet aggregation was inactivated by sulfate conjugation. This observation suggested that conjugated form of compounds **2**, **3** and **5** might be inactive. Accordingly, it might be difficult to directly correlate these observations with the effectiveness of this drug against the "Oketsu" syndrome, because the precise information regarding the pharmacokinetics and pharmacodynamics in-vivo of these metabolites as a conjugated and/or free form is unclear. Further detailed study is necessary to clarify the differences of activity between the free and conjugated forms. However, our studies indicated that deconjugated compounds **2**–**5** partly contributed to the anti-Oketsu effect of Shimotsu-To through the inhibition of the platelet aggregation and our results are consistent with the well-known empirical properties of this drug in traditional Chinese medicine.

The sulfate is inactive and so variations in the clinical response to compounds **2**–**5** between individuals may be due to differences in the endogenous level of the free form. Differing amounts of sulfotransferase or differences in isoenzymes may explain the effectiveness of this drug against platelet aggregation.

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