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# Quantitation of Chafurosides A and B in Tea Leaves and Isolation of Prechafurosides A and B from Oolong Tea Leaves

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A procedure was developed for the quantitative determination of chafuroside A, a flavone *C*-glycoside with potent anti-inflammatory activity, and its regioisomer chafuroside B, as well as isovitexin and vitexin, by selected reaction monitoring liquid chromatography−tandem mass spectrometry (SRM LC-MS/MS) analysis. This method was successfully applied to commercial leaves of green tea, houji tea, oolong tea, and black tea. High levels of chafurosides A and B were found in oolong tea leaves that had been heated at >140 °C. Next, their precursors, prechafurosides A and B, were isolated from methanol extract of oolong tea leaves prepared from Shizu 7132, *Camellia sinensis* (L.) O. Kuntze, by partition with *n*-butanol and $H_2O$ and chromatography on Diaion SP-825, Sephadex LH-20, and ODS C-18, guided by assay of chafuroside formation. Prechafurosides A and B gave chafurosides A and B, respectively, in good yields when heated at 160 °C for 0.5 h. Solvolysis of prechafurosides A and B with pyridine and dioxane quantitatively afforded isovitexin and vitexin, respectively. On the basis of these results and physicochemical data (MS, UV, and NMR), prechafurosides A and B were concluded to be new flavone *C*-glycoside sulfates, isovitexin-2″-sulfate and vitexin-2″-sulfate, respectively.

**KEYWORDS: Oolong tea; tea leaf; chafuroside A; chafuroside B; isovitexin; vitexin; isovitexin-2″-sulfate; vitexin-2″-sulfate**

## INTRODUCTION

Tea is made from evergreen leaves of *Camellia sinensis* and is the most widely consumed beverage after water in daily life worldwide. The common commercial types are green tea, oolong tea, pu-erh tea, and black tea. The processing methods of their leaves are similar, except for the manner of fermentation and firing. The fermentation level increases from unfermented green tea to partially fermented oolong tea to well-fermented black tea (*1*). Firing involves heating at high temperature by means of a hot plate, hot air, or flame after drying, and some species of green tea and oolong tea leaves are subjected to strong firing at > 160 °C to enhance stability, taste, and flavor.

There has been increasing interest in polyphenolic compounds contained in foodstuffs, especially in tea leaves, because of their beneficial effect on human health. Uehara et al. (*2*) showed that daily drinking of oolong tea (1 L) for 4 weeks improved skin condition in 62% of patients with atopic dermatitis. We recently isolated new flavone *C*-glycosides with a condensed dihydrofuran ring, chafurosides A and B (**Figure 1**), from oolong tea (unpublished information) by means of fractionation guided by assay of anti-inflammatory activity in an atopic dermatitis model (*3*).

Many biologically active polyphenolic compounds, such as EGCG and theaflavins, have been isolated from green and black tea leaves (*4*−*6*). There have been fewer studies on oolong tea leaves (*7*, *8*). Interestingly, we found that oral administration of chafuroside A prevents skin inflammation in an ICR mouse atopic disease model at the dose of 10 μg/kg, which is much lower than the effective doses of prednisolone and betamethasone (10 and 0.8 mg/kg, respectively). Like indomethacin, chafuroside A reduced AOM-induced development of colon aberrant crypt foci in rats and APC-deficient mice at 10 ppm (*9*).

Furanoflavonoids show various biological activities, including anti-inflammatory, antifungal, antiviral, and/or antibacterial activities. A representative structure of this type of flavonoid is flavone with a furan ring fused at C-7,8, such as lamceolatin (*10*). In contrast, flavone *C*-glycosides with a furan ring between sugar and aglycone are rare. Ketohexofuranosides have both *C*- and *O*-glycosidic linkages at the same carbon (C2) of hexose, forming a 5,5-spiroketal ring system. Recently, pinnatifinosides A and B were reported to have unusual glycoside structures, including β-D-allofuranose and α-D-allofuranose, respectively (*11*). To our knowledge, however, the coexistence of *C*- and *O*-glycoside linkages at separate positions in chafurosides A and B is novel.

We considered that chafuroside A is a promising candidate as an anti-inflammatory and chemopreventive agent. Only about 1 mg of chafuroside A was isolated from 3.2 kg of commercial oolong tea leaves, so it is important to develop a method for quantitative determination of chafurosides to identify teas with high levels of this chemical, both for biosynthetic and metabolic studies and for therapeutic use. In the present study, we developed

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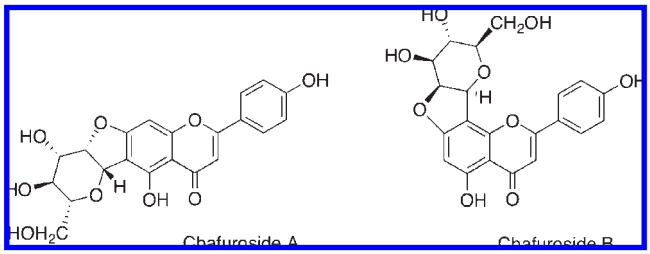



**Figure 1.** Structures of chafurosides A and B from oolong tea.

a method for quantitative determination of chafurosides A and B, together with their structural analogues, isovitexin and vitexin, respectively, by using ion-spray LC-MS/MS and applied it to analyze green tea, houji tea, oolong tea, and black tea from *C. sinensis*. Here, we report on the concentrations of chafurosides in various tea leaves, as well as the isolation, structural determination, and reactivity of chafuroside precursors in oolong tea leaves.

## MATERIALS AND METHODS

**General.** $^{1}H$ and $^{13}C$ spectra were obtained on a JEOL ECA-500 spectrometer at 500 and 125 MHz, respectively, with chemical shifts being reported as $\delta$ (ppm) from tetramethylsilane as an internal standard. $^{1}H-^{1}H$ COSY, $^{1}H-^{13}C$ COSY, and HMBC spectra were obtained with the JEOL standard pulse sequences, and data processing was performed with standard software Delta. IR and UV spectra were taken on a JASCO WS/IR-8000 grating infrared spectrometer and on a Hitachi UV absorption spectrometer U-2900. Optical rotation was measured with a JASCO DIP-1000 digital polarimeter.

LC-MS/MS measurements were performed with triple-stage quadrupole instruments equipped with electrospray ionization (ESI) interfaces: API 3000 system (Applied Biosystems, Foster City, CA). Analyst software (version 1.4.1) was used for data acquisition and analysis. QTOF MS and MS/MS measurements were performed with QSTAR Elite (Applied Biosystems) equipped with electrospray ionization (ESI) interfaces.

ESI-MS and MS/MS analyses for compound identification were performed on a Q-Star XL Hybrid LC-MS/MS System (Applied Biosystems, Foster City, CA) using an Agilent 1200 series binary HPLC pump with a 50 mm × 2.1 mm i.d., 3 $\mu$m, Inertsil ODS-SP column (GL Sciences, Tokyo, Japan). For system control and data acquisition we used Analyst QS software.

**Samples and Reagents.** Commercial leaves of green tea, Yabukita and Sayamakaori, houji tea of Yabukita, oolong tea, Shikisyu and Suisen, and black tea, Darjeeling and Assam, were used. Green tea, houji tea, oolong tea, and black tea leaves were prepared from fully grown tea leaves of Shizu 7132, *C. sinensis* (L.) O. Kuntze, in summer 2007 according to the traditional manners described below. The temperatures of green tea, oolong tea, and black tea leaves were strictly controlled under 90 °C in the courses of their processing. Houji tea leaves were prepared by roasting the green tea leaves, thus obtained, on a hot plate at around 190−200 °C for 4 min.

All HPLC or analytical grade solvents were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Authentic isovitexin and vitexin were isolated from Puriri (*Vitex lucens*) in New Zealand, using the purification procedures described for flavone *C*-glycoside. Authentic chafurosides A and B were synthesized from isovitexin and vitexin, respectively, by means of modified Mitsunobu reaction with triphenylphosphine and diethyl azodicarboxylate (*12*, *13*). Sep-Pak plus C18 cartridges were purchased from Waters Corp. (Milford, MA), Diaion SP 825 from Mitsubishi Kagaku (Tokyo, Japan) and Sephadex LH-20 from GE Healthcare Bio-Science AB (Uppsala, Sweden).

**LC-ESI-MS/MS Analysis.** *Conditions. (1) ESI-MS/MS.* In both positive and negative modes, full-scan, single MS mass spectra were acquired over the mass range of $m/z$ 300−1200 by direct infusion of four standards in $H_2O$/MeOH eluent (flow rate = 0.2 mL/min).

Full-scan collision-induced dissociation (CID) spectra were acquired by colliding the quadrupole 1 (Q1) selected precursor ion at an argon pressure of approximately $1.3 \times 10^{-4}$ mbar in the collision cell (Q2) and scanning the second quadrupole mass spectrometer (Q3) in the range of $m/z$ 80−500.

A precursor ion for CID and diagnostic product ion for the target were identified to carry out selective reaction monitoring (SRM) LC-MS/MS analysis. The source and desolvation temperatures were maintained at 100 and 300 °C, respectively. The desolvation and cone gas flows were set at 680 and 0 L/h. The electrospray capillary voltage and cone voltage were optimized for each standard.

*(2) Liquid Chromatography.* Liquid chromatography was performed on an Agilent 1100 LC system (Agilent Technologies, Waldbronn, Germany). Chromatographic separation of chafurosides A and B, isovitexin, and vitexin was carried out on a 150 mm × 3 mm i.d., 3 $\mu$m, Cadenza CD-C18 column (Intact, Kyoto, Japan). The column temperature was kept at 45 °C. The mobile phase reservoirs contained (A) $H_2O$/MeOH (40:60) for the analysis of authentic standards. HPLC conditions were as follows: 100% A for 20 min to elute vitexin, isovitexin, and chafurosides A and B and then a linear gradient to 100% MeOH at 22 min, hold for 8 min to wash the column, return to initial conditions in 31 min, and condition column for 9 min before the next injection. The flow rate from 0 to 20 min was 0.3 mL/min and that from 20 to 40 min was 0.5 mL/min.

*Quantitative Determination of Chafurosides A and B, Isovitexin, and Vitexin by LC-MS/MS.* Ten microliters of the test sample from each type of tea leaves (0.1 g) in 0.5−10 mL of 50% MeOH was used for LC-MS/MS analyses of chafurosides A and B, isovitexin, and/or vitexin. Reference standard solution (5 $\mu$L) was injected before and after each sample analysis. The peak area was used for calibrating flavone *C*-glycoside amounts. The determination limit was taken to be the amount giving a signal-to-noise ratio of 5.

*Recovery Rates of Chafurosides A and B, Isovitexin, and Vitexin.* Recoveries of chafurosides A and B, isovitexin, and/or vitexin were determined at concentrations of 5, 50, and 500 ng/g, respectively. The $H_2O$-soluble fraction from 100% MeOH extract of green tea leaves from Shizu 7132 was used in this experiment. The $H_2O$-soluble fraction was spiked with the standards and extracted with *n*-BuOH ($n$ = 3). The *n*-BuOH-soluble fraction was evaporated to dryness, then the residue was dissolved in 50% MeOH, and this solution was subjected to LC-MS/MS analysis.

*Preparation of Tea Leaf Extract and Its Fractionation.* Leaves (0.5 g) of commercial green tea, houji tea (roasted green tea), oolong tea, and black tea and those prepared from Shizu 7132 were ground and extracted twice with $H_2O$, 30% MeOH, or 100% MeOH (5.0 mL) under reflux for 40 min. Each extract was partitioned between *n*-BuOH (1 mL × 2) and $H_2O$ (1 mL). The extract, $H_2O$-soluble fraction, and *n*-BuOH-soluble fraction were subjected to LC-MS/MS analysis for quantitation of chafurosides A and B, isovitexin, and vitexin.

*Heat Treatment of Tea Leaves, Extraction, and Fractionation.* Leaves of commercial green tea, houji tea, oolong tea, and black tea and those prepared from Shizu 7132, their extracts, and the fractions derived from these extracts were placed in glass tubes and heated in an oven (Nihon Buchi K.K., Tokyo, Japan).

**Isolation of Chafuroside Precursors from Tea Leaves.** Oolong tea leaves prepared from Shizu 7132 (1 kg) were ground and extracted twice with MeOH under reflux for 40 min. The extract (348 g) was partitioned between *n*-BuOH and $H_2O$. The aqueous fraction (142 g) was chromatographed on Diaion SP 825 with $H_2O$, 30% MeOH, and 100% MeOH to obtain fractions 1−3, respectively. Fractions 1 and 2 afforded substantial amounts of chafurosides on heating at 160 °C.

Fraction 2 gave an active fraction, fraction 4 (1.97 g), eluted between $K_d$ = 2.30 and 2.80 on Sephadex LH-20 using MeOH. Successive chromatography of fraction 4 on ODS-C18 (Sep-Pak C18-Gel) afforded active fractions 5 (87 mg) and 6 (63 mg). Finally, purification of both fractions on ODS HPLC using Cadenza CD-C18 afforded the chafuroside A and B precursors, prechafuroside A (**1**) and its regioisomer, prechafuroside B (2) (6 and 4 mg), respectively. Compound **1** gave chafuroside A, and compound **2** gave chafuroside B in good yields, respectively, when heated at 160 °C.

During the isolation process, all eluates were monitored by means of chafuroside formation assay (heating at 160 °C).

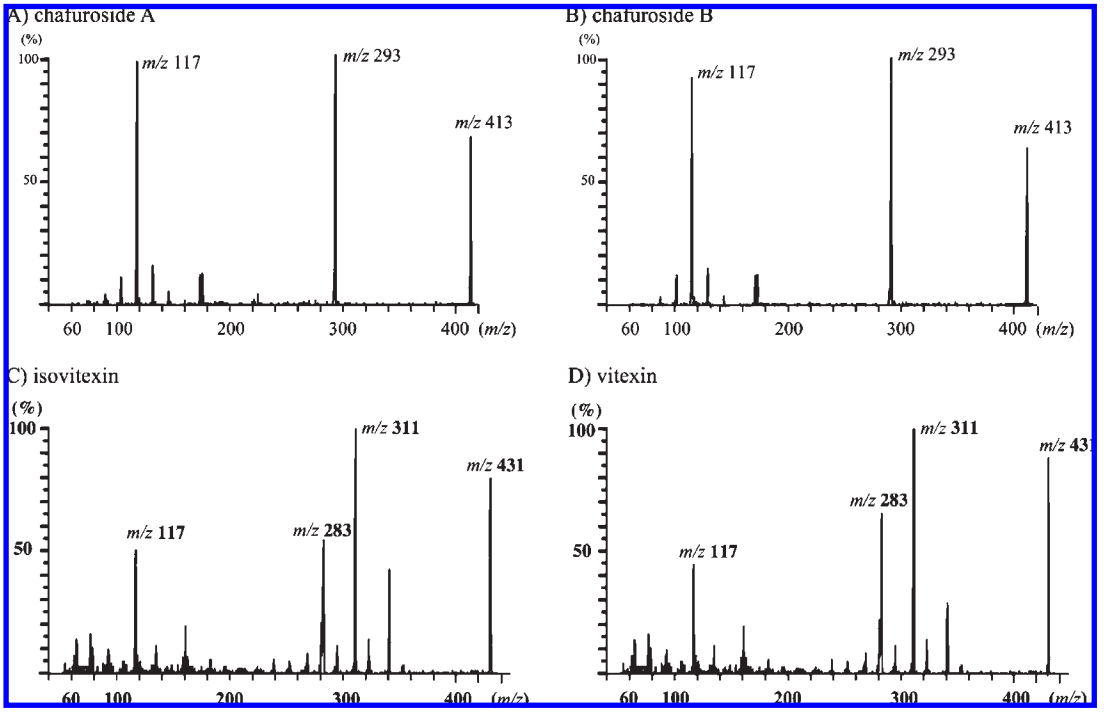



**Figure 2.** Negative product ion mass spectra of (**A**) chafuroside A and (**B**) chafuroside B with the $[M - H]^-$ ions at *m/z* 413 and 413 and of (**C**) isovitexin and (**D**) vitexin at *m/z* 431 and 431 as precursors, respectively.

Chafuroside A (**1**) was obtained as a light yellow powder: $[\alpha]_D^{26}$ = −45.0° (*c* 0.6, MeOH); UV $\lambda_{max}$ (MeOH) nm (log $\varepsilon$) 330 (4.34) and 273 (4.39); ESI-MS *m/z* 511 $[M - H]^-$; HR-QTOFMS *m/z* 511.05612 $[M - H]^-$; HR-FABMS *m/z* 511.0550 $[M - H]^-$ (calcd for $C_{21}H_{20}O_{13}S - H$, 511.05625); IR $\nu_{max}$ (neat) 3600−3120, 1653, 1608, 1354, 1242, 1201,1178, and 812 $cm^{-1}$; $^1H$ and $^{13}C$ NMR data in dimethyl sulfoxide (DMSO)-$d_6$ are shown in **Table 6**.

Chafuroside B (**2**) was obtained as a light yellow powder: $[\alpha]_D^{26}$ = −23.0° (*c* 0.6, MeOH); UV $\lambda_{max}$ (MeOH) nm (log $\varepsilon$) 326 (4.24) and 270 (4.31); ESI-MS *m/z* 511 $[M-H]^-$. HR-QTOFMS *m/z* 511.05643 $[M-H]^-$ (HR-FABMS *m/z* 511.0551 $[M - H]^-$ (calcd for $C_{21}H_{20}O_{13}S - H$, 511.05625); IR $\nu_{max}$ (neat) 3580−3100, 1655, 1610, 1356, 1244, 1200, 1180, and 814 $cm^{-1}$; $^1H$ and $^{13}C$ NMR data in DMSO-$d_6$ are shown in **Table 6**.

**Conditions for the Acquisition of TOFMS and MS/MS Spectra.** HPLC conditions were as follows. Acetonitrile (10%) was delivered as the mobile phase at 0.2 mL/min. The column temperature was kept at 40 °C. ESI-TOFMS and ESI-Q-TOFMS were conducted in the negative product ion scan mode; the ion spray voltage was set at −4500 V. Dry air (GS1) was maintained at 75 psi. The declustering potentials **1** and **2** were set at −50 and −15 V, respectively. On ion-dependent acquision (IDA) for MS and MS/MS analyses of compounds **1** and **2**, the collision energy was set at −5 and −20 eV, respectively. Mass spectrometer calibration was performed with a mixture of CsI ($[M - H]^-$, *m/z* 126.90503), taurocholic acid ($[M - H]^-$, *m/z* 514.28440), and synthetic peptide iPD1 (ALILTLVS) ($[M - H]^-$, *m/z* 827.52477).

## RESULTS AND DISCUSSION

**Chafuroside Analysis with LC-MS/MS.** *Optimization of LC-ESI-MS/MS Conditions.* Negative ion MS of chafurosides A and B, isovitexin, and vitexin standard solution was initially implemented with $H_2O$/MeOH or $H_2O$/acetonitrile (1:1) as the mobile phase, which promoted the selective and highly sensitive formation of $[M - H]^-$ of chafurosides A and B at *m/z* 413 and of isovitexin and vitexin at *m/z* 431, as well as gave good chromatographic performance during LC-MS analyses. In further CID-MS/MS experiments, typical negative ion mass spectra (range *m/z* 300−500) of chafurosides A and B, isovitexin, and vitexin standard solutions were obtained (**Figure 2**). Significant fragment ions were observed at *m/z* 293 and 117 in the cases of chafurosides A and B and at *m/z* 341, 311, 283, and 117 in the cases of isovitexin and vitexin. The product ions at *m/z* 293 of the former compounds showed the highest intensity at a collision energy of −36 eV and a capillary voltage of −4.0 kV, and the product ions at *m/z* 311 of the latter compounds showed the highest intensity at a collision energy of −32 eV and a capillary voltage of −4.0 kV.

To achieve maximum selectivity, as well as quantitative analysis, SRM was then implemented. On the basis of the MS/MS fragmentations of chafurosides A and B, isovitexin, and vitexin, combination precursor−product ions of *m/z* 413−293 for chafurosides A and B and of *m/z* 431−311 for isovitexin and vitexin were considered to be suitable for confirmatory analysis. Typical chromatograms from the SRM LC-MS/MS analyses of authentic standard chafuroside A, chafuroside B, isovitexin, and vitexin (100 ng/mL) are shown in **Figure 3**. Good linearities ($R^2 > 0.99$) of the peak areas with increasing amounts of the four standards were confirmed over the range from 0.005 to 1 ng per injection (0.5−100 ng/mL of $H_2O$-soluble fraction of the MeOH extract of oolong tea (0.1 g)). The determination limits of chafurosides A and B, isovitexin, and vitexin in the spiked $H_2O$-soluble fraction part, based on a signal-to-noise ratio of 5, were 0.01, 0.01, 0.02, and 0.02 ng/g of the $H_2O$-soluble fraction from MeOH extract of oolong tea, respectively.

*Recoveries of Chafurosides A and B, Isovitexin, and Vitexin.* Recoveries of chafurosides A and B, isovitexin, and vitexin, spiked at three dose levels, from the $H_2O$-soluble fraction of the MeOH extract of oolong tea leaves were 85−105, 80−112, 83−98, and 96−113%, respectively (**Table 1**).

*Analyses of Green Tea, Oolong Tea, and Black Tea Leaves.* The MeOH extracts from tea leaf samples were examined using the SRM LC-MS/MS method (**Figure 3**). The chromatographic peaks eluted at the retention times of the authentic standards showed that relatively large amounts of isovitexin and vitexin (ng/g of tea leaves) were contained in the MeOH extracts

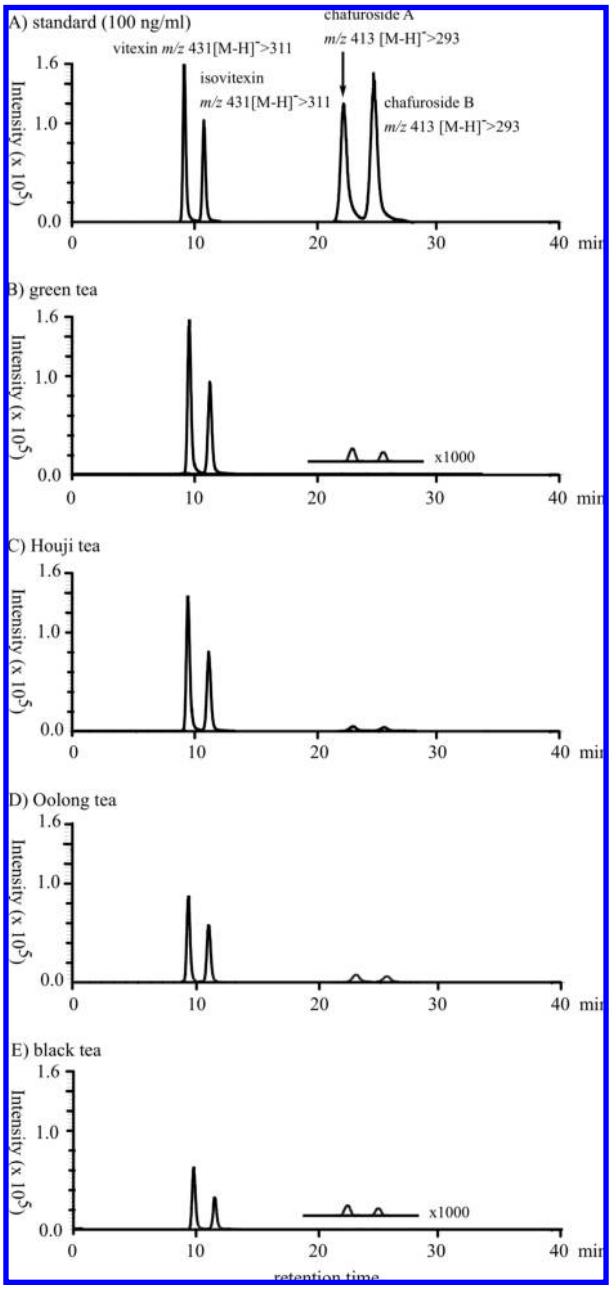



**Figure 3.** Selected reaction monitoring liquid chromatography−tandem mass spectrometry chromatograms of authentic standards (100 ng/mL) (**A**) and 100% MeOH extracts (mg/mL) from green tea and houji tea leaves from Shizu 7132 (**B**, **C**), oolong tea Suisen (**D**) and Darjeeling black tea (**E**) with negative ESI. Precursor−product ion combinations and polarity used in SRM detection are shown.

**Table 1.** Recovery of Chafurosides A and B, Isovitexin, and Vitexin from Spiked $H_2O$-Soluble Fraction of 100% MeOH Extract of Oolong Tea Leaves from Shizu 7132[a]

| | recovery (%) of | | | |
|---|---|---|---|---|
| concn (ng/g) | chafuroside A | chafuroside B | isovitexin | vitexin |
| 5 | 85.3 ± 7.1 | 80.4 ± 5.9 | 83.6 ± 9.3 | 95.5 ± 7.9 |
| 50 | 105.2 ± 12.9 | 112.8 ± 8.5 | 98.3 ± 8.1 | 113.5 ± 6.9 |
| 500 | 90.0 ± 9.9 | 87.5 ± 7.2 | 85.8 ± 3.1 | 94.5 ± 9.9 |

[a] Results are mean (±SE) ($n$ = 3).

**Table 2.** Contents of Chafurosides A and B, Isovitexin, and Vitexin in 100% MeOH Extracts from Commercial Tea Leaves[a]

| | contents (ng/g of tea leaves) of | | | |
|---|---|---|---|---|
| *classification*/variety | chafuroside A | chafuroside B | isovitexin | vitexin |
| *green tea* | | | | |
| Shiizu 7132 | 52 ± 4 | 42 ± 7 | 102110 ± 3040 | 88390 ± 2940 |
| Yabukita | 38 ± 2 | 24 ± 3 | 96440 ± 2840 | 89430 ± 2770 |
| Sayamakaori | 34 ± 6 | 24 ± 1 | 88800 ± 2250 | 74570 ± 2580 |
| *houji tea* | | | | |
| Shiizu 7132 | 4980 ± 170 | 3650 ± 350 | 87060 ± 2370 | 184470 ± 2560 |
| Yabukita-1 | 1980 ± 150 | 1770 ± 100 | 64520 ± 1910 | 60950 ± 1790 |
| Yabukita-2 | 3140 ± 240 | 2590 ± 200 | 44540 ± 1610 | 51440 ± 1640 |
| *oolong tea* | | | | |
| Shiizu 7132 | 50 ± 1 | 39 ± 2 | 76700 ± 2210 | 72620 ± 2290 |
| Suisen | 8580 ± 760 | 7960 ± 360 | 32340 ± 970 | 28790 ± 710 |
| Shikisyu | 690 ± 70 | 640 ± 60 | 41750 ± 890 | 40210 ± 790 |
| *black tea* | | | | |
| Shiizu 7132 | 78 ± 2 | 53 ± 2 | 65580 ± 1490 | 59890 ± 1350 |
| Assam | 18 ± 2 | 23 ± 2 | 22580 ± 570 | 19880 ± 450 |
| Darjeeling | 97 ± 9 | 72 ± 9 | 20640 ± 440 | 19000 ± 420 |

[a] Results are mean (±SE) ($n$ = 3).

**Table 3.** Contents of Chafurosides A and B in 100% MeOH Extracts from Oolong Tea Leaves Prepared from Shizu 7132 after Heating at Various Temperatures for 40 min[a]

| | contents (ng/g of tea leaves) of compound after heating at | | | |
|---|---|---|---|---|
| compound | 140 °C | 160 °C | 180 °C | 200 °C |
| chafuroside A | 780 ± 16 | 8650 ± 750 | 7760 ± 670 | 172 ± 10 |
| chafuroside B | 556 ± 14 | 7260 ± 710 | 7140 ± 650 | 169 ± 9 |

[a] Results are mean (±SE) ($n$ = 3).

**Table 4.** Contents of Chafurosides A and B in 100% MeOH Extract of Oolong Tea Leaves Prepared from Shizu 7132 after Heating at 160 °C

| | contents of compound (ng/g of tea leaves) after heating for | | | |
|---|---|---|---|---|
| compound | 20 min | 40 min | 60 min | 80 min |
| chafuroside A | 3480 ± 216 | 7580 ± 750 | 8790 ± 770 | 8870 ± 610 |
| chafuroside B | 2244 ± 224 | 6950 ± 610 | 7580 ± 640 | 7990 ± 590 |

Results are mean ±SE ($n$ = 3).

(**Table 2**), as reported previously (*14*). The mean values were highest in green tea and houji tea and lowest in oolong tea and black tea. As noted above, the fermentation levels are different among these teas, and this result indicates that isovitexin and vitexin are gradually oxidized during oxidative fermentation in the processing of oolong tea and black tea.

The leaves of green tea, houji tea, and black tea also contained chafurosides A and B. Contents of these compounds in houji tea and oolong tea (except oolong tea prepared from Shizu 7132) were at similar levels, whereas the mean values of green tea and black tea were also similar, but 100 times lower than those of houiji tea and oolong tea. These results suggest that putative precursors of chafurosides remain intact during the preparation processes of green tea, houji tea, and oolong tea.

Traditionally green tea leaves are prepared as follows. Fresh young tea leaves are steamed quickly after having been picked and are then cooled and dried stepwise with rolling by hand on a hot plate at around 40−80 °C. Black tea leaves are processed by oxidative fermentation of young tea leaves by means of gradual

drying at room temperature for several hours with thorough rolling or after cutting, followed by rapid drying with hot air (at around 100 °C). Houji tea leaves are prepared from the green tea by roasting at 160−200 °C for several minutes. Oolong tea leaves are produced from fully grown tea leaves by semifermentation under sunlight for a few hours, followed by shaking on a bamboo tray in a room, and then heated quickly at around 160−260 °C. They are finally dried at around 70−80 °C. Some species of oolong tea are further subjected to additional strong firing to intensify the taste and flavor. The common feature in the preparation of houji and oolong tea leaves is strong firing. Interestingly, oolong and houji tea leaves that contained large amounts of chafurosides A and B showed signs of burning. Thus, heating at high temperature is assumed to be important for the production of chafurosides.

**Table 5.** Contents of Chafurosides A and B, Isovitexin, and/or Vitexin in 100% MeOH, 50% MeOH, and $H_2O$ Extracts of Oolong Tea Leaves Prepared from Shizu 7132 and Their $H_2O$- and *n*-BuOH-Soluble Fractions Obtained by Partition of These Extracts with *n*-BuOH and $H_2O$ before and after Heating at 160 °C for 40 min[a]

| | | contents (ng/g of tea leaves) of compound | | |
|---|---|---|---|---|
| extraction solvent | compound | extract | *n*-BuOH fraction | $H_2O$ fraction |
| | | Before Heating | | |
| 100% MeOH | chafuroside A | 41 ± 1 | 38 ± 1 | − |
| | chafuroside B | 33 ± 1 | 30 ± 1 | − |
| | isovitexin | 92870 ± 2760 | 82760 ± 2550 | − |
| | vitexin | 79480 ± 2450 | 73450 ± 2640 | − |
| 50% MeOH | chafuroside A | 50 ± 1 | 47 ± 1 | − |
| | chafuroside B | 39 ± 2 | 35 ± 2 | − |
| $H_2O$ | chafuroside A | 52 ± 1 | 49 ± 1 | − |
| | chafuroside B | 45 ± 1 | 41 ± 1 | − |
| | | After Heating | | |
| 100% MeOH | chafuroside A | 7660 ± 440 | 35 ± 1 | 6580 ± 437 |
| | chafuroside B | 6950 ± 410 | 29 ± 1 | 5850 ± 480 |
| | isovitexin | 85480 ± 2450 | 75880 ± 2390 | − |
| | vitexin | 77090 ± 2250 | 69660 ± 1940 | − |
| 50% MeOH | chafuroside A | 12550 ± 770 | 42 ± 1 | 10180 ± 740 |
| | chafuroside B | 14290 ± 660 | 33 ± 1 | 11580 ± 780 |
| $H_2O$ | chafuroside A | 13820 ± 780 | 47 ± 1 | 11270 ± 660 |
| | chafuroside B | 14850 ± 710 | 41 ± 1 | 12540 ± 700 |

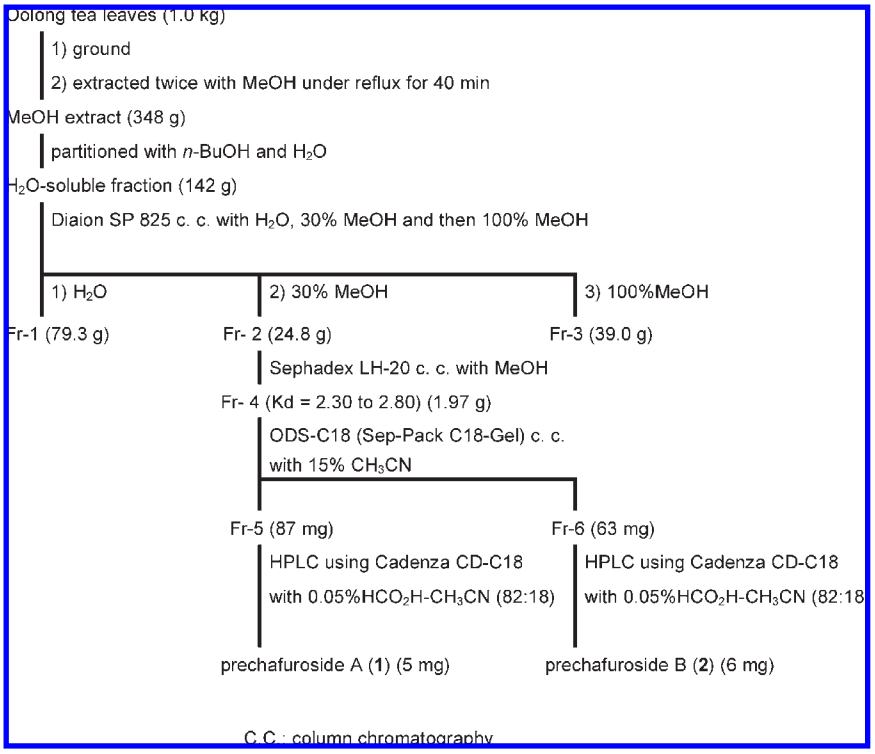
[a] Results are mean ± SE (*n*=3). −, not detected.

*Extraction and Fractionation of Chafurosides and Their Precursors*. The contents of chafurosides A and B in MeOH extract from oolong tea leaves prepared from Shizu 7132 were drastically increased after heating at 160−180 °C (**Tables 2** and **3**). The contents did not increase substantially when heating was prolonged over about 40 min (**Tables 2** and **4**). Thus, chafurosides are produced by heating oolong tea leaves at around 160−180 °C for about 40 min.

Chafurosides A and B, isovitexin, and vitexin were detected in MeOH extracts and their *n*-BuOH-soluble fractions, but not in the $H_2O$-soluble fractions (**Table 5**). The contents of chafurosides A and B in all of the extracts and their $H_2O$-soluble fractions were drastically increased to similar levels after heating at 160 °C for 40 min. Interestingly, the contents of chafurosides in *n*-BuOH-soluble fraction were similar before and after heating. These results indicate that the precursors of chafurosides A and B are not isovitexin and vitexin, but are more hydrophilic.

**Isolation and Structure Determination of Chafuroside A and B Precursors, Prechafurosides A and B, from Oolong Tea Leaves from Shizu 7132.** *Isolation of Prechafurosides A and B*. Isolation of prechafurosides A (**1**) and B (**2**), in fraction 2 from MeOH extract of oolong tea leaves prepared from Shizu 7132 was achieved in four steps of column chromatography (Diaion SP 825, Sephadex LH-20, ODS-C18, and HPLC), as shown in **Chart 1**. Fraction 1 contains other precursors of both chafurosides. Isolation of these compounds is in progress.

**Chart 1.** Isolation of Prechafuroside A (**1**) and Prechafuroside B (**2**) from Oolong Tea Leaves from Shizu 7132, *Camellia sinensis* (L.) O. Kuntze



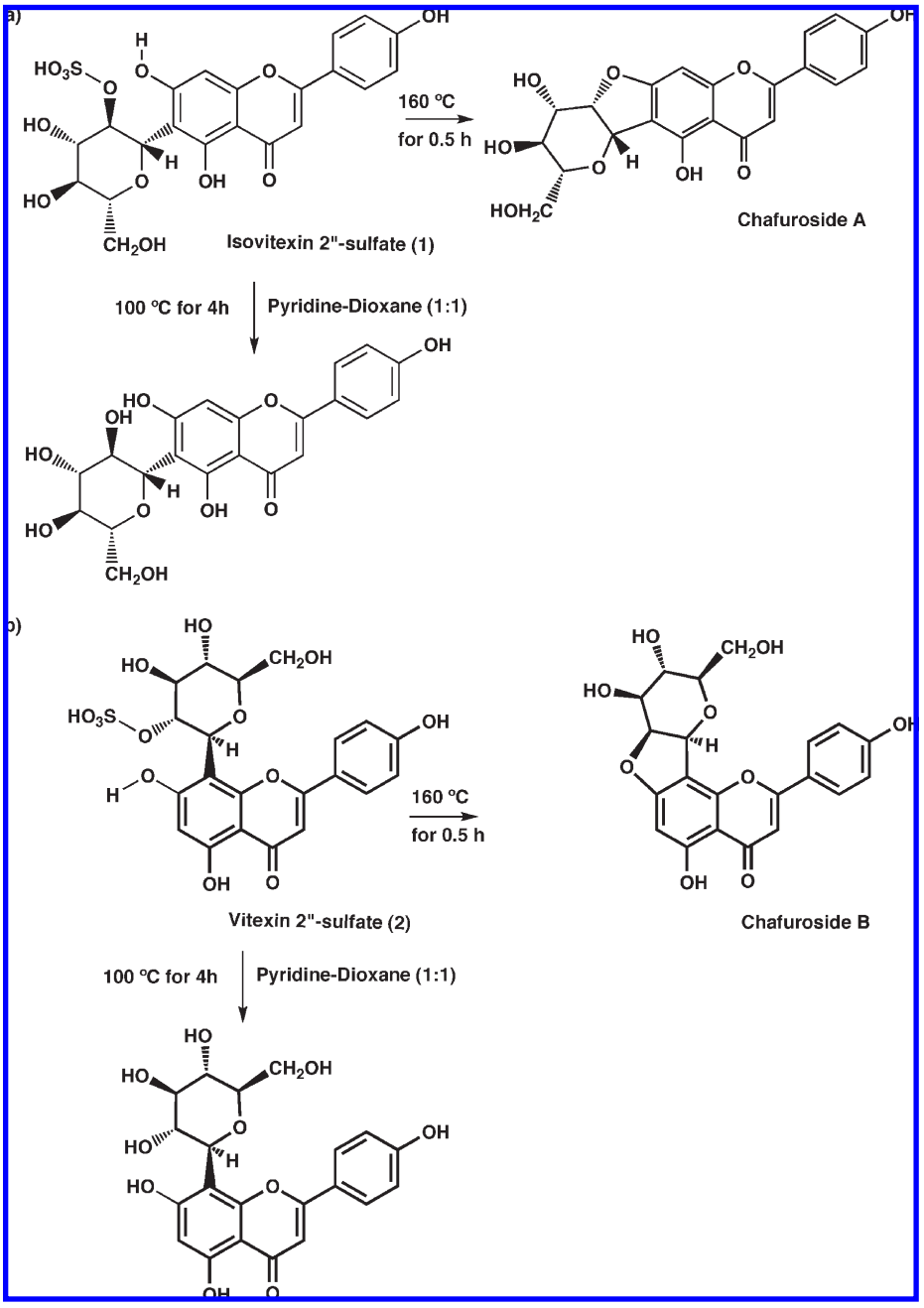



**Figure 4.** Preparation of (**a**) chafuroside A and isovitexin from isovitexin 2″-sulfate (**1**) and (**b**) chafuroside B and vitexin from vitexin 2″-sulfate (**2**).

*Structure Determination of Prechafurosides A and B.* The precursors of chafurosides A and B, **1** and **2**, have not yet been crystallized. The IR spectra of compounds **1** and **2** suggested the presence of hydroxyl, conjugated carboxyl, and sulfate ester functions in both molecules. The UV characteristics of **1** and **2** are similar to those of isovitexin and vitexin, respectively (*15*).

As illustrated in **Figure 4**, compounds **1** and **2** quantitatively afforded isovitexin and vitexin, respectively, on solvolysis with pyridine−dioxane. Compounds **1** and **2** gave chafuroside A and chafuroside B, respectively, in good yields when heated at 160 °C. The $^{1}H$ and $^{13}C$ NMR data of **1**, isovitexin, **2**, and vitexin are shown in **Table 6**. There were no significant differences in the chemical shifts of the corresponding carbons and protons, except for those of the C-2″ carbons and H-2″ protons in the glucose moieties, between **1** and isovitexin and between **2** and vitexin. Increases of the chemical shifts of the C-2″ carbons and protons of **1** and **2** by 5.1 and 0.86 ppm and by 5.7 and 0.80 ppm compared with those of isovitexin and vitexin, respectively, indicated that the hydroxyl groups at C-2″ of the two former compounds were sulfated, on the basis of similar findings in heparin derivatives (*16*).

On the basis of these data and other NMR (2D-COSY, C−H shift COSY, and HMBC) spectra of prechafurosides A and B, prechafuroside A was concluded to be isovitexin 2″-*O*-sulfate and prechafuroside B to be vitexin 2″-*O*-sulfate.

The proposed structures were well supported by negative ion Q-TOF (MS/MS) experiments (complete product scan) carried out on the $[M - H]^-$ ion ($m/z$ 511) of **1** and **2** (**Figure 5**). Bond cleavage between C-2″ and O-2″-$SO_3H$ was evidenced by ion formation of $m/z$ 96.9548 from **1** and of $m/z$ 96.9601 (calcd. for $HSO_4$, $m/z$ 96.9596) from **2**. Other prominent ions, $m/z$ 431.1041 from **1** and $m/z$ 431.0969 (calcd. for $C_{21}H_{29}O_{10}$, $m/z$ 431.0978) from **2**, were generated by characteristic bond cleavage between O-2″ and $SO_3H$.

**Table 6.** $^{1}H$ and $^{13}C$ NMR Data for Prechafuroside A, Isovitexin, Prechafuroside B, and Vitexin in DMSO-$d_6$[a]

| | isovitexin | | prechafuroside A | | vitexin | | prechafuroside B | |
|---|---|---|---|---|---|---|---|---|
| no. | $\delta_C$ | $\delta_H$(mult, *J* in Hz) | $\delta_C$ | $\delta_H$(mult, *J* in Hz) | $\delta_C$ | $\delta_H$(mult, *J*) | $\delta_C$ | $\delta_H$(mult, *J* in Hz) |
| 1 | | | | | | | | |
| 2 | 163.3 | | 163.4 | | 162.4 | | 162.7 | |
| 3 | 102.8 | 6.78 (s) | 102.8 | 6.77 (s) | 102.4 | 6.52 (s) | 102.9 | 6.72 (s) |
| 4 | 182.0 | | 182.0 | | 182.0 | | 182.7 | |
| 5 | 156.2 | | 156.3 | | 155.9 | | 155.8 | |
| 6 | 108.9 | | 108.8 | | 98.1 | 6.20 (s) | 98.4 | 6.19 (s) |
| 7 | 163.5 | | 163.3 | | 163.9 | | 164.3 | |
| 8 | 93.6 | 6.50 (s) | 94.0 | 6.44 (s) | 104.0 | | 103.3 | |
| 8a | 161.2 | | 161.1 | | 160.3 | | 161.0 | |
| 4a | 103.4 | | 103.6 | | 104.5 | | 104.5 | |
| 1' | 121.1 | | 121.2 | | 121.5 | | 121.8 | |
| 2' | 128.5 | 7.93 (d, 8.5) | 128.9 | 7.90 (d, 8.5) | 128.8 | 8.01 (d, 8.5) | 128.9 | 8.00 (d, 8.5) |
| 3′ | 116.0 | 6.91 (d, 8.5) | 116.0 | 6.90 (d, 8.5) | 115.7 | 6.89 (d, 8.8) | 115.5 | 6.86 (d, 8.5) |
| 4' | 160.7 | | 160.0 | | 161.0 | | 161.4 | |
| 5′ | 116.0 | 6.91 (d, 8.5) | 116.0 | 6.90 (d, 8.5) | 115.7 | 6.89 (d, 8.8) | 115.5 | 6.86 (d, 8.5) |
| 6' | 128.5 | 7.93 (d, 8.5) | 128.9 | 7.90 (d, 8.5) | 128.8 | 8.01 (d, 8.5) | 128.9 | 8.00 (d, 8.5) |
| 1″ | 73.0 | 4.57 (d, 10.0) | 70.6 | 4.64 (d, 9.5) | 73.3 | 4.68 (d, 10.0) | 71.1 | 4.74 (d, 10.0) |
| 2″ | 70.6 | 4.03 (dd, 10.0, 9.0) | 75.7 | 4.54 (dd, 9.5, 9.0) | 70.8 | 3.84 (dd, 10.0, 9.0) | 76.5 | 4.64 (dd, 10.0, 9.0) |
| 3″ | 78.9 | 3.14 (dd, 9.2, 9.0) | 78.3 | 3.39 (dd, 9.2, 9.0) | 78.6 | 3.28 (dd, 9.2, 9.0) | 78.4 | 3.49 (dd, 9.2, 9.0 ) |
| 4″ | 70.2 | 3.12 (dd, 9.2, 9.0) | 70.3 | 3.15 (dd, 9.2, 9.0) | 70.5 | 3.39 (dd, 9.2, 9.0) | 71.0 | 3.45 (dd, 9.2, 9.0) |
| 5″ | 81.6 | 3.10 (ddd, 9.0, 5.0, 2.5) | 81.0 | 3.11 (ddd, 9.0, 5.0, 2.5) | 81.7 | 3.24 (ddd, 9.0, 5.1, 2.5) | 81.6 | 3.29 (ddd, 9.0, 5.1, 2.5) |
| 6″a | 61.5 | 3.67 (dd, 12.0, 2.5) | 61.5 | 3.70 (dd, 11.5, 2.5) | 61.3 | 3.76 (dd, 12.2, 2.5) | 61.5 | 3.74 (dd, 11.0, 2.5) |
| 6″b | | 3.38 (dd, 12.0, 5.0) | | 3.41 (dd, 11.5, 5.0) | | 3.54 (dd, 12.2, 5.1) | | 3.54 (dd, 11.0, 5.1) |

[a] Assignments were based on $^{1}H-^{1}H$-COSY, $^{1}H-^{13}C$-COSY, and HMBC experiments.

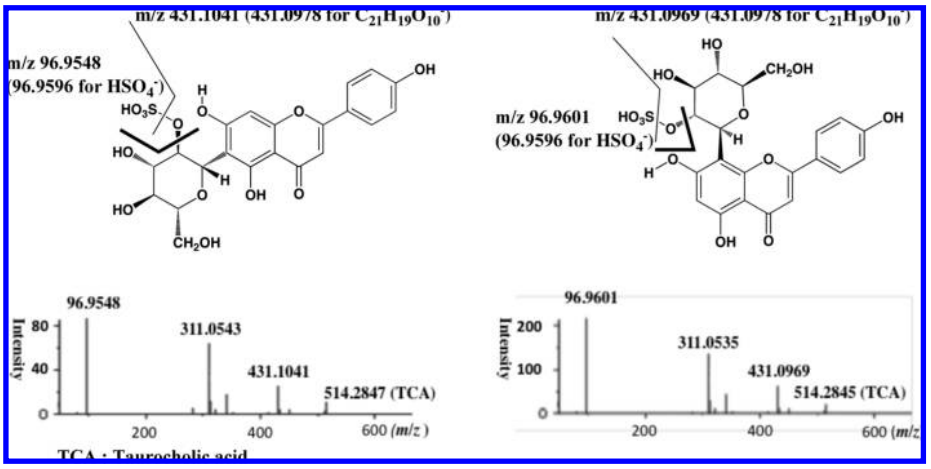



**Figure 5.** Negative MS/MS spectra of (**A**, left) isovitexin 2″-sulfate (**1**) and (**B**, right) vitexin 2″-sulfate (**2**).

These data allowed us to assign the structures of prechafurosides A and B as isovitexin 2″-sulfate and vitexin 2″-sulfate, respectively.

**Formation Mechanism of Chafurosides A and B from Prechafurosides A and B.** It is reported that a sulfonyl group and an adjacent free hydroxyl group in trans configuration on a sugar unit in a polysaccharide undergo desulfation and subsequent cyclization to give a relatively stable intermediate containing an oxirane ring between C-2 and C-3, when a slightly alkaline solution of heparin is simply freeze-dried (*17*, *18*). This alkali-catalyzed 2-O-desulfation and simultaneous etheric bond formation between C-2 and C-3 are considered to involve an $S_N2$ mechanism via a transition state in which an anion formed by deprotonation of O-3 attacks C-2. Formation of chafurosides A and B by heating under neutral conditions from their precursors **1** and **2** may similarly involve an $S_N2$ mechanism via a transition state in which the anion formed by deprotonation of O-7 attacks C-2″ (**Figure 6**).

To our knowledge, this is the first report of the isolation of flavone *C*-glycoside sulfate from oolong tea leaves.

In conclusion, procedures for quantitative determination of chafurosides A and B, isovitexin, and/or vitexin by liquid chromatography−tandem mass spectrometry were developed and successfully applied to leaves of green tea, houji tea, oolong tea, and black tea. Isovitexin and vitexin were detected in all tea leaves, although only trace levels were found in one sample of oolong tea leaves. We established an isolation procedure for precursors of chafurosides A and B in oolong tea leaves from Shizu 7132 and identified them as flavone *C*-glycoside sulfates, isovitexin 2″-sulfate (**1**) and vitexin 2″-sulfate (**2**), respectively,

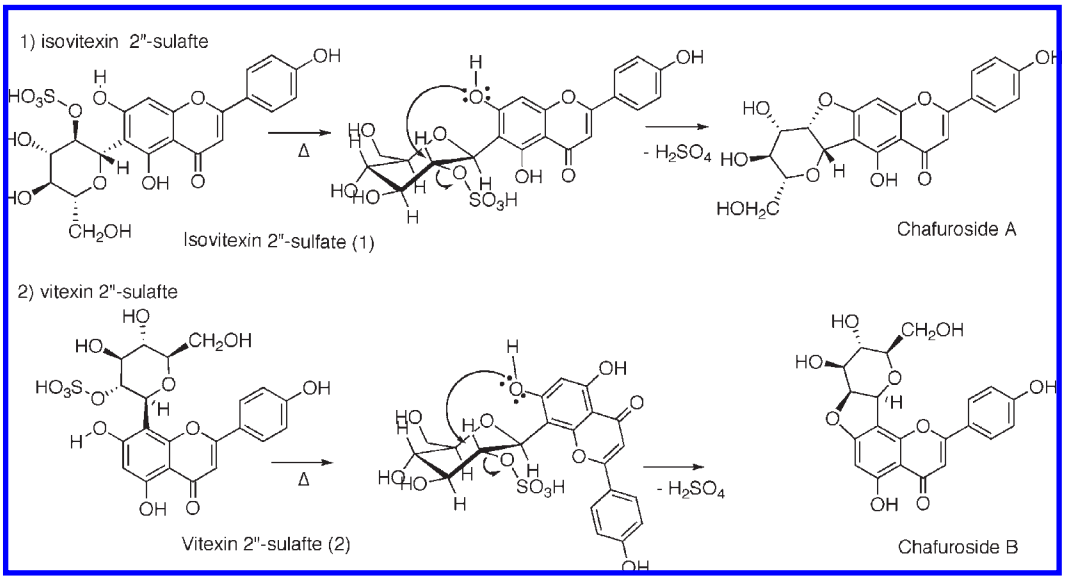



**Figure 6.** Proposed formation mechanisms of etheric bond in isovitexin 2″-sulfate (**1**) and vitexin 2″-sulfate (**2**).

on the basis of chemical and physicochemical data. Study on the formation mechanism of prechafurosides A and B in the oolong tea leaves is ongoing.

Oolong tea rich in chafurosides may ameliorate atopic dermatis, and our quantitation methods could be useful for monitoring chafuroside content during the processing of tea leaves, as well as for identifying tea species and other plants rich in chafuroside precursors.

## LITERATURE CITED

(1) Kuroda, Y.; Hara, Y. Antimutagenic and anticarcinogenic activity of tea polyphenols. *Mutat. Res.* **1999**, *436*, 69–97.

(2) Uehara, M.; Sugiura, H.; Sakurai, K. A trial of oolong tea in the management of recalcitrant atopic dermatitis. *Arch. Dermatol.* **2001**, *137*, 143–148.

(3) Kuehl, F. A.; Humes, J. L.; Egan, R. W.; Ham, E. A.; Beveridge, G. C.; Van Arman, C. G. Role of prostaglandin endoperoxide PGG2 in inflammatory processes. *Nature (London)* **1977**, *265*, 170–173.

(4) Nagle, D. G.; Ferreira, D.; Zhou, Y.-D. Epigallocatechin-3-gallate (EGCG): chemical and biomedical perspectives. *Phytochemistry* **2006**, *67*, 1849–1855.

(5) Higdon, J. V.; Frei, B. Tea catechins and polyphenols: health effects, metabolism, and antioxidant functions. *Crit. Rev. Food Sci. Nutr.* **2003**, *43*, 89–143.

(6) Zaveri, N. T. Green tea and its polyphenolic catechins: medicinal uses in cancer and noncancer applications. *Life Sci.* **2006**, *78*, 2073–2080.

(7) Nakai, M.; Fukui, Y.; Asami, S.; Toyoda-Ono, Y.; Iwashita, T.; Shibata, H.; Mitsunaga, T.; Hashimoto, F.; Kiso, Y. Inhibitory effects of oolong tea polyphenols on pancreatic lipase *in vitro*. *J. Agric. Food Chem.* **2005**, *53*, 4593–4598.

(8) Hashimoto, F.; Nonaka, G.; Nishioka, I. Tannins and related compounds. XC. 8-*C*-ascorbyl (−)-epigallocatechin 3-*O*-gallate and novel dimeric flavan-3-ols, oolonghomobisflavans A and B, from oolong tea. (3). *Chem. Pharm. Bull.* **1989**, *37*, 3255–3263.

(9) Niho, N.; Mutoh, M.; Sakano, K.; Takahashi, M.; Hirano, S.; Nukaya, H.; Sugimura, T.; Wakabayashi, K. Inhibition of intestinal carcinogenesis by a new flavone derivative, chafuroside, in oolong tea. *Cancer Sci.* **2006**, *97*, 248–251.

(10) Yadav, P. P.; Ahmed, G.; Maruya, R. Furanoflavonoids from *Pongamia pinnata* fruits. *Phytochemistry* **2004**, *65*, 439–443.

(11) Maurya, R.; Yadav, P. P. Furanoflavonoids: an overview. *Nat. Prod. Rep.* **2005**, *22*, 400–424.

(12) Furuta, T.; Kimura, T.; Kondo, S.; Mihara, H.; Wakimoto, T.; Nukaya, H.; Tsuji, K.; Tanaka, K. Concise total synthesis of flavone *C*-glycoside having potent anti-inflammatory activity. *Tetrahedron* **2004**, *42*, 9375.

(13) Nakatsuka, T.; Tomimori, Y.; Fukuda, Y.; Nukaya, H. First total synthesis of structurally unique flavonoids and their strong anti-inflammatory effect.. *Bioorg. Med. Chem. Lett.* **2004**, *14*, 3201.

(14) Engelhardt, U. H.; Finger, A.; Kuhr, S. Determination of flavone C-glycosides in tea. *Z. Lebensm. Unters. Forsch.* **1993**, *197*, 239–244.

(15) Manki; Tomimori, T.; Ito, M. Constituents of *Swertia japonica*. I. Structures of swertisin and isoswertisin. *Chem. Pharm.Bull.* **1967**, *15*, 263–269.

(16) Yates, E. A.; Santini, F.; Cristofano, D. B.; Payre, N.; Cosentino, C.; Guerrini, M.; Naggi, A.; Torri, G.; Hricovini, M. Effect of substitution pattern on $^{1}$H, $^{13}$C NMR chemical shifts and $^{1}$JCH coupling constants in heparin derivatives. *Carbohydr. Res.* **2000**, *329*, 239–247.

(17) Jaseja, M.; Rej, R. N.; Sauriol, F.; Perlin, A. S. Novel regio- and stereoselective modifications of heparin in alkaline solution. Nuclear magnetic resonance spectroscopic evidence. *Can. J. Chem.* **1989**, *67*, 1449–1456.

(18) Ishihara, M.; Kariya, Y.; Kikuchi, H.; Minamisawa, T.; Yoshida, K. Importance of 2-*O*-sulfate groups of uronate residues in heparin for activation of FGF-1 and FGF-2. *J. Biochem.* **1997**, *121*, 345–349.

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