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Polyphenol Composition of a Functional Fermented Tea Obtained by Tea-Rolling Processing of Green Tea and Loquat Leaves

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Supporting Information

ABSTRACT: Phenolic constituents of a new functional fermented tea produced by tea-rolling processing of a mixture (9:1) of tea leaves and loquat leaves were examined in detail. The similarity of the phenolic composition to that of black tea was indicated by high-performance liquid chromatography comparison with other tea products. Twenty-five compounds, including three new catechin oxidation products, were isolated, and the structures of the new compounds were determined to be (2*R*)-2-hydroxy-3-(2,4,6-trihydroxyphenyl)-1-(3,4,5-trihydroxyphenyl)-1-propanone 2-*O*-gallate, dehydrotheasinensin H, and acetonyl theacitrin A by spectroscopic methods. In addition, theacitrinin A and theasinensin H were obtained for the first time from commercial tea products. Isolation of these new and known compounds confirms that reactions previously demonstrated by in vitro model experiments actually occur when fresh tea leaves are mechanically distorted and bruised during the production process.

KEYWORDS: Catechin, oxidation, tea fermentation, black tea, polyphenol, *Camellia sinensis*, *Eriobotrya japonica*

INTRODUCTION

Leaves of the tea plant [*Camellia sinensis* (L.) Kuntze], are processed to various nonfermented and fermented tea products.[1] Green tea is a nonfermented tea, consumed mainly in East Asia. The phenolic constituents are similar to those of fresh tea leaves, which are mainly composed of epigallocatechin, epicatechin, and their galloyl esters. In contrast, black tea is a typical fermented tea and contains various catechin oxidation products, such as theaflavins and theasinensins.[2] The term "fermentation" is industry-specific jargon used traditionally in tea manufacturing, and microbial processes are not involved in the production process. During the tea fermentation process, chemical changes in tea leaf constituents are catalyzed by tea leaf enzymes under controlled conditions. Recently, we have developed a new fermented tea, produced by tea-rolling processing of a mixture of green tea and loquat (*Eriobotrya japonica*) leaves (MT),[3] and demonstrated notable biological activities, such as hypotriacylglycerolemic and antiobesity properties[4] and suppression of blood glucose levels.[5] The production procedure includes a characteristic process: Partially dried green tea leaves are mixed with a small amount of fresh loquat leaves (1/9 of tea leaves by weight) and mechanically distorted and bruised with a tea-rolling machine. The leaves are then heated and dried to terminate the enzymatic reaction. The procedure does not involve the tea fermentation process that is the most important step in typical black tea manufacture. Instead, during the tea-rolling process of the MT production, oxidation of the tea catechins with tea leaf enzymes is accelerated by the addition of loquat leaf enzymes. Furthermore, acceleration is enhanced by coupling the oxidation mechanism with chlorogenic acid, which is one of the major polyphenols in loquat leaves. The mechanism is summarized as follows: Chlorogenic acid is oxidized by enzymes to a corresponding *ortho*-quinone preferentially due to its higher enzyme specificity. The resulting quinone acts as a strong oxidant to oxidize tea catechins to catechin quinones, and simultaneously, the quinone of chlorogenic acid is reduced to the original chlorogenic acid.[6,7] The catechin quinones undergo nonenzymatic complex coupling reactions with other catechin molecules to give black tea polyphenols.[8] Polyphenols of loquat leaves were not detected in the tea products, except for a small amount of oligomeric procyanidins.[6] The total polyphenol content of the loquat leaves is much lower than that of green tea (about 1/5), and the major constituents were shown to be epicatechin, procyanidins, caffeoyl quinic acids, and flavonoid glycosides,[9] some of which are commonly found in tea leaves. In addition, the amount of the loquat leaves used for production of MT is only 1/9 the weight of the fresh tea leaves. Therefore, the composition of the phenolic compounds of MT is basically similar to that of normal black tea.[6] In the present paper, we compare the polyphenol composition of MT with those of other tea products. In addition, detailed investigation of the chemical constituents leads to the isolation of catechin oxidation

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products, which are important in the chemistry of catechin oxidation in the production of uncharacterized fermented tea polyphenols.

MATERIALS AND METHODS

Materials. MT was produced from leaves of *C. sinensis* var. *sinensis* cv. Yabukita and loquat leaves (9:1, w/w) at Nagasaki Agricultural and Forestry Technical Development Center, Higashisonogi Tea Research Station, in July 2009. One of the two green tea products was produced from the same tea leaf at the same location. The other green tea product was purchased in a local market in Hangzhou, China. Oolong tea (Ti Kuan Yin, Fujian, China, and Shui Hsien, Guangdong, China) and black tea (Darjeeling, India, and Uva, Sri Lanka) were purchased at a local market in Nagasaki, Japan. Standard samples of tea catechins were isolated from green tea and purified by crystallization from H_2O. Theasinensins, theaflavins, and other black tea polyphenols were prepared by enzymatic oxidation of tea catechins.[7,10] A standard sample of the polymeric polyphenols was obtained by further purification of a sample separated in a previous study:[6] The polymeric polyphenols were subjected to Diaion HP20SS column chromatography, where the column was first eluted with 50% MeOH to wash out hydrophilic portions of the polymers. Subsequently, the relatively hydrophobic fractions used as the standard were eluted with 80% MeOH.

Analytical Procedures. UV spectra were obtained with a JASCO V-560 UV/vis spectrophotometer, and optical rotations were measured with a JASCO DIP-370 digital polarimeter (JASCO Co., Tokyo, Japan). ^{1}H, ^{13}C NMR, ^{1}H−^{1}H correlation spectroscopy, heteronuclear single quantum coherence, and heteronuclear multiple bond correlation (HMBC) spectra were measured on a Varian UNITY plus 500 (500 MHz for ^{1}H and 125 MHz for ^{13}C NMR) (Varian, Palo Alto, CA) and a JEOL JNM-AL400 (400 MHz for ^{1}H and 100 MHz for ^{13}C NMR) spectrometer (JEOL Ltd., Tokyo, Japan). Coupling constants are expressed in Hz, and chemical shifts are given on a δ (ppm) scale. MS were recorded on a Voyager DE-PRO (Applied Biosystems, Foster City, CA) and a JEOL JMS-700N spectrometer. 2,5-Dihydroxybenzoic acid and *m*-nitrobenzyl alcohol were used as the matrix for matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) and fast atom bombardment (FAB)-MS measurements, respectively. Elemental analysis was conducted with a PerkinElmer 2400 II analyzer (PerkinElmer Inc., Waltham, MA). Column chromatography was performed using Diaion HP20 (Mitsubishi Chemical Co. Tokyo, Japan), Sephadex LH-20 (25−100 μm, GE Healthcare Bio-Science AB, Uppsala), Chromatorex ODS (100−200 mesh; Fuji Silysia Chemical Ltd., Tokyo, Japan), Toyopearl HW-40C (75 μm; Tosoh Bioscience Japan, Tokyo, Japan), and Wakogel 100C18 (63-212 μm; Wako Pure Chemical Industries, Osaka, Japan) columns. TLC was performed on precoated Kieselgel 60 F_{254} plates (0.2 mm thick; Merck, Darmstadt, Germany) with toluene−ethyl formate−formic acid (1:7:1, v/v) or $CHCl_3$−MeOH−H_2O (14:6:1, v/v) as a solvent, and spots were detected by UV illumination (254 nm) and by spraying with 2% ethanolic $FeCl_3$ and 10% sulfuric acid reagent, followed by heating.

High-Performance Liquid Chromatography (HPLC) Analysis of Tea Polyphenols. Tea polyphenols were analyzed by a modified method described in a previous study.[6] Tea products were separately pulverized, and a portion (0.50 g) was extracted with 60% EtOH containing 0.1% of trifluoroacetic acid (TFA) (15 mL) at room temperature in a shaking incubator for 2 days. After filtration, an aliquot (5.0 μL) was analyzed by HPLC. The HPLC was performed on a Cosmosil 5C_{18}-ARII (Nacalai Tesque, Kyoto, Japan) column (250 mm × 4.6 mm i.d., 5 μm) with gradient elution from 4 to 30% (39 min) and from 30 to 75% (15 min) CH_3CN in 50 mM H_3PO_4; flow rate, 0.8 mL/min; temperature, 35 °C; and detection, JASCO photodiode array detector MD-2010. Theaflavins and polymeric polyphenols were analyzed as follows: The above-mentioned extract was filtered through a membrane filter (0.45 μm), and the filtrate (10.0 mL) was concentrated to dryness by rotary evaporator. The residue was partitioned between water (4.0 mL) and a mixture of EtOAc and hexane (4:1, v/v, 4.0 mL). The aqueous layer was further extracted with the same organic solvent two times. The organic layer was combined and concentrated to dryness. The residue was dissolved to 60% EtOH containing 0.1% TFA (5.0 mL), and a portion (10.0 μL) was analyzed for determination of theaflavin composition. The HPLC conditions were as follows: Cosmosil 5C_{18}-PAQ (Nacalai Tesque, Kyoto, Japan) column (250 mm × 4.6 mm i.d., 5 μm) with gradient elution from 10 to 25% (5 min) and 25 to 80% (40 min) CH_3CN in 50 mM H_3PO_4; flow rate, 0.8 mL/min; and detection at UV 375 nm (theaflavin, t_R = 26.3 min; theaflavin-3-*O*-gallate, t_R = 29.9 min; theaflavin-3′-*O*-gallate, t_R = 31.9 min; and theaflavin-3,3′-di-*O*-gallate, t_R = 33.2 min). The final aqueous layer was concentrated to dryness and dissolved in 60% EtOH containing 0.1% TFA (10.0 mL). An aliquot (5.0 μL) was analyzed for determination of polymeric polyphenols. The HPLC conditions were as follows: Cosmosil 5C_{18}-PAQ column (250 mm × 4.6 mm i.d., 5 μm) with gradient elution from 10 to 23% (28 min), 25 to 80% (2 min), and then isocratic elution at 80% (10 min) CH_3CN in 50 mM H_3PO_4; flow rate, 1.0 mL/min; and detection at UV 270 nm (polymeric polyphenols: t_R = 33.4 min) (see the Supporting Information). The analyses were carried out in quadruplicate.

Separation of Polyphenols. Dried MT (1.5 kg) was crushed in a Waring (Torrington, CT) blender and extracted with acetone−H_2O (6:4, v/v, 18 L) at room temperature three times, and the extract was concentrated under reduced pressure. The resulting aqueous solution was mixed with MeOH (about 20% of the total volume) to dissolve insoluble polyphenols and applied to a Sephadex LH-20 (10 cm × 35 cm) column with 20% MeOH. After the column was washed with 20% MeOH, the column was eluted with 40−100% MeOH (10% stepwise elution, each 1 L) and then 60% acetone. The fractions containing polyphenols were combined, and the organic solvent was removed by evaporation under reduced pressure. The aqueous solution (2 L) was partitioned with EtOAc (2 L) five times, and the EtOAc layer (104 g) was separated by Sephadex LH-20 column chromatography (6.5 cm × 48 cm) with H_2O−MeOH (0−100% MeOH, 10% stepwise elution, each 0.5 L) and then 60% acetone to give five fractions. Fraction 2 (11.5 g) was subjected to Diaion HP20 column chromatography (5.5 cm × 25 cm) with 0−100% MeOH to give gallic acid (293 mg), theasinensin C (85 mg), and subfractions 2-3 to 2-16. Chromatography of fraction 2-3 (900 mg) over Sephadex LH-20 (3.2 cm × 33 cm) with EtOH yielded (−)-epigallocatechin (**7**) (500 mg) and (+)-gallocatechin (89 mg). Fraction 2-5 (1.34 g) was successively separated by Sephadex LH-20 (3 cm × 33 cm) with EtOH and Toyopearl HW-40C (3 cm × 17 cm) with EtOH to yield **7** (393 mg), 1,6-di-*O*-galloyl-β-D-glucose (53 mg), and (−)-epicatechin (330 mg). Fraction 2-7 (1.47 g) was applied to a Sephadex LH-20 column with EtOH and then Chromatorex ODS column (3 cm × 17 cm) with 0−30% MeOH (5% stepwise) to give (−)-epicatechin (756 mg) and procyanidin B2 (60 mg). Fraction 2-10 (260 mg) was purified by Chromatorex ODS (3 cm × 17 cm) with 0−35% MeOH (5% stepwise, each 200 mL) and preparative HPLC using Wakogel 100C18 to give acetonyl theacitrin A (**5**) (24.3 mg). Fraction 2-12 (945 mg) was crystallized from aqueous MeOH to yield myricetin-3-*O*-glucoside (60 mg). Fraction 2-15 (940 mg) was separated by Chromatorex ODS chromatography (3 cm × 23 cm) with 30−55% MeOH followed by crystallization from MeOH to give quercetin-3-*O*-galactoside (75 mg) and quercetin-3-*O*-glucoside (79 mg). Fraction 3 (43.1 g) was further fractionated by Diaion HP20 column chromatography (8 cm × 42 cm) with 0−100% MeOH (10% stepwise elution, each 0.5 L) to give 10 subfractions. Fraction 3-3 (2.72 g) was separated by a combination of column chromatography using Chromatorex ODS (0−40% MeOH), Sephadex LH-20 (EtOH), Wakogel 100C18 (0−20% MeOH), and MCI-gel CHP20P (0−40% MeOH) to

yield theasinensin H (**2**) (15.0 mg), theasinensin B (**8**) (706 mg), (+)-catechin (55.2 mg), procyanidin B4 (99.0 mg), 1-*O*-galloyl-*β*-D-glucose (1.6 mg), strictinin (20.9 mg), 2,3,5,7-tetrahydroxychroman-3-*O*-gallate (73.5 mg), 2(*R*)-2-hydroxy-3-(2,4,6-trihydroxyphenyl)-1-(3,4,5-trihydroxyphenyl)-propanone 2-*O*-gallate (**3**) (21.5 mg), and (−)-epigallocatechin-3-*O*-gallate (**6**) (49.5 mg). Similar chromatographic separation of fraction 3-4 (10.1 g) afforded theaflavonin (123.7 mg), 1,4,6-tri-*O*-galloylglucose (38.7 mg), and **6** (7.5 g). Purification of fraction 3-5 (9.6 g) with Sephadex LH-20 chromatography (EtOH) afforded (−)-epicatechin-3-*O*-gallate (5.6 g). Fraction 4 (19.5 g) contained **6**, (−)-epicatechin-3-*O*-gallate, and theasinensin A and was not examined in this study. Fraction 5 (23.5 g) was separated by Diaion HP20 chromatography (5 cm × 30 cm) with 0−100% MeOH (10% stepwise elution) to give theasinensin A (2.4 g), a mixture of theaflavins (14.2 g), and a subfraction fraction 5-2 (4.3 g). Further separation of the subfraction by chromatography using Sephadex LH-20 (80% MeOH) and Chromatorex ODS (0−40% MeOH) yielded theacitrinin A (**1**) (54.8 mg) and dehydrotheasinensin H (**4**) (125.8 mg).

Theasinensin H (2). Brown amorphous powder, $[\alpha]_D^{27}$ −80.9° (*c* 0.1, MeOH). FAB-MS *m*/*z*: 763 $[M + H]^+$. HR-FAB-MS *m*/*z*: 763.1497 $[M + H]^+$ ($C_{37}H_{31}O_{18}$ requires 763.1509). UV λ_{max}^{MeOH} nm (log *ε*): 209 (4.97), 275 (4.03), 381 (2.87). IR ν_{max} cm^{-1}: 3393, 1685, 1617, 1517, 1457. ^{1}H NMR (400 MHz, in acetone-d_6 + D_2O) *δ*: 7.35 (1H, s, B-6), 7.05 (2H, s, galloyl-2,6), 6.98 (1H, s, B-6′), 5.98, 5.96, 5.92, 5.85 (each 1H, d, *J* = 2.0, 2.4, 2.4, 2.0 Hz, respectively, A-8, A-8′, A-6′, A-6), 5.14 (1H, br s, C-3), 4.89 (1H, br s, C-2), 4.59 (1H, br s, C-2′), 4.21 (1H, br s, C-3′), 2.77 (1H, br d, *J* = 17.6 Hz, C-4′), 2.56 (1H,br d, *J* = 16.6 Hz, C-4), 2.54 (1H, dd, *J* = 4.4, 16.6 Hz, C-4), 2.53 (1H, dd, *J* = 4.9, 17.6 Hz, C-4′). ^{13}C NMR (100 MHz, in acetone-d_6 + D_2O) *δ*: 166.7 (galloyl-7), 157.6, 157.5 (2C), 157.4, 157.2, 157.1 (A-5, 7, 8a, A′-5, 7, 8a), 146.0 (2C) (galloyl-3, 5), 145.7 (B′-5), 145.4 (B-5), 143.4 (B′-3),143.3 (B-3), 138.9 (galloyl-4), 133.9 (B′-4), 133.4 (B-4), 130.9 (B′-1), 129.6 (B-1), 121.4 (galloyl-1), 114.3 (B′-2), 113.8 (B-2), 110.0(2C) (galloyl-2, 6), 109.3 (B′-6), 108.5 (B-6), 99.6 (C′-4a), 98.5 (C-4a), 96.2, 96.0, 95.6, 95.5 (A-6, 8, A′-6, 8), 77.3 (C′-2), 76.2 (C-2), 68.1 (C-3), 65.0 (C′-3), 30.4 (C′-4), 27.4 (C-4).

Hydrolysis of 2. A solution of **2** (2 mg) in H_2O (1 mL) was stirred with tannase (2 mg) at room temperature for 12 h, and the products were analyzed by HPLC. The retention time (t_R) and UV absorption at 9.15 min coincided with those of an authentic sample of theasinensin E.[11] The t_R of theasinensin C, the atrop isomer with *R*-biphenyl bond, was 7.37 min.

(2*R*)-2-Hydroxy-3-(2,4,6-trihydroxyphenyl)-1-(3,4,5-trihydroxyphenyl)-1-propanone 2-*O*-gallate (3). Tan amorphous powder, $[\alpha]_D^{25}$ −88.2° (*c* 0.2, MeOH). FAB-MS *m*/*z*: 475 $[M + H]^+$. UV λ_{max}^{MeOH} nm (log *ε*): 209 (4.97), 275 (4.03), 381 (2.87). IR ν_{max} cm^{-1}: 3383, 1689, 1665, 1608, 1533. Anal. calcd for $C_{22}H_{18}O_{12}3H_2O$: C, 50.01; H, 4.58. Found: C, 50.34; H, 4.51. ^{1}H NMR (400 MHz, in acetone-d_6 + D_2O) *δ*: 7.38 (2H, s, H-2′, 6′), 7.18 (2H, s, galloyl H-2, 6), 6.17 (1H, dd, *J* = 3.4, 10.3 Hz, H-2), 6.01 (2H, s, H-3″, 5″), 3.25 (1H, dd, *J* = 10.3, 14.0 Hz, H-3), 3.14 (1H, dd, *J* = 3.4, 14.0 Hz, H-3). ^{13}C NMR (100 MHz, in acetone-d_6 + D_2O) *δ*: 195.6 (C-1), 166.4 (galloyl C-7), 158.1(2C) (C-2″, 6″),, 157.9 (C-4″), 146.1 (C-3′, 5′), 145.8(2C) (galloyl C-3, 5), 139.3 (galloyl C-4), 138.6 (C-4′), 127.4 (C-1′), 121.9 (galloyl C-1), 110.3(2C) (galloyl C-2, 6), 109.4 (C-2′, 6′), 102.8 (C-1″), 95.6(2C) (C-3″, 5″), 76.1 (C-2), 26.6 (C-3).

Dehydrotheasinensin H (4). Brown amorphous powder, $[\alpha]_D^{27}$ −49.9° (*c* 0.1, MeOH). MALDI-TOF-MS *m*/*z*: 783 $[M + Na]^+$. Anal. calcd for $C_{37}H_{28}O_{18}\cdot 5H_2O$: C, 52.24; H, 4.50. Found: C, 51.72; H, 4.65. UV λ_{max}^{MeOH} nm (log *ε*): 210 (4.84), 275 (4.15), 384 (3.75). IR ν_{max} cm^{-1}: 3396, 1651, 1606, 1506, 1456. ^{1}H NMR (400 MHz, in acetone-d_6) *δ*: 7.08 (1H, d, *J* = 0.8 Hz, B′-6), 6.79 (2H, s, galloyl-2, 6), 6.14 (1H, d, *J* = 2.2 Hz, A-6), 6.11 (1H, br s, C′-2), 6.09, 6.04, 5.96 (each 1H, d, *J* = 2.2, 2.1, 2.1 Hz, respectively, A-8, A′-6, A′-8), 5.94 (1H, br s, C′-3), 4.58 (1H, br s, C-3), 4.06 (1H, br s, C-2), 3.16 (1H, dd, *J* = 2.8, 17.5 Hz, C′-4), 2.96 (1H, d, *J* = 11.3 Hz, B-6), 2.75 (1H, br d, *J* = 17.5 Hz, C′-4), 2.93 (1H, br d, *J* = 15.9 Hz, C-4), 2.81 (1H, dd, *J* = 4.7, 15.9 Hz, C-4), 2.33 (1H, d, *J* = 11.3 Hz, B-6). ^{13}C NMR (100 MHz, in acetone-d_6) *δ*: 189.6 (B-4), 166.2 (galloyl-7), 157.6 (A-5), 157.6 (A-7), 157.4 (A′-5), 156.9 (A′-8a), 155.5 (A-8a), 152.2 (B′-3), 151.0 (B′-5), 145.7(2C) (galloyl-3, 5), 139.7 (B-3), 138.7 (galloyl-4), 134.3 (B-2), 130.8 (B′-1), 129.4 (B′-4), 121.3 (galloyl-1), 112.4 (B′-2), 110.2 (B′-6), 109.9 (2C) (galloyl-2,6), 100.2 (A′-4a), 98.5 (A-4a), 96.5 (A-6), 96.2 (A-8), 95.7 (A′-6), 95.4 (A′-8), 94.7 (B-5), 89.6 (B-1), 76.8 (C′-2), 72.2 (C-2), 66.4 (C′-3), 65.0 (C-3), 40.7 (B-6), 28.3 (C′-4), 25.0 (C-4).

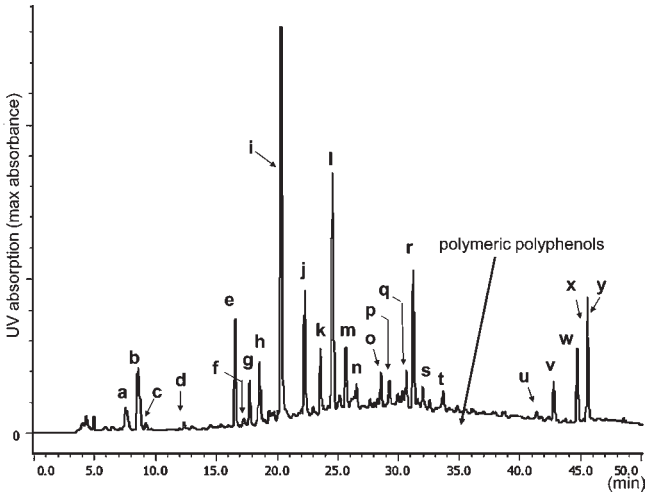


Figure 1. HPLC profile (max absorbance) of 60% EtOH extract of MT. Peaks: a, theasinensin C; b, gallic acid; c, theogallin; d, theobromine; e, theasinensin B (**8**); f, theasinensin H (**2**); g, (−)-epigallocatechin (**7**); h, 1,6-di-*O*-galloyl-*β*-D-glucose; i, caffeine; j, theasinensin A; k, (−)-epicatechin; l, (−)-epigallocatechin-3-*O*-gallate (**6**); m, (−)-gallocatechin-3-*O*-gallate; n, apigenin-C-glucoside; o, quercetin 3-*O*-*β*-D-glucosyl-(1→3)-α-L-rhamnosyl-(1→6)-*β*-D-galactoside; p, quercetin 3-*O*-*β*-D-glucosyl-(1→3)-α-L-rhamnosyl-(1→6)-*β*-D-glucoside; q, kaempferol 3-*O*-*β*-D-glucosyl-(1→3)-α-L-rhamnosyl-(1→6)-*β*-D-galactoside; r, (−)-epigallocatechin-3-*O*-gallate; s, kaempferol 3-*O*-*β*-D-glucosyl-(1→3)-α-L-rhamnosyl-(1→6)-*β*-D-glucoside; t, dehydrotheasinensin AQ; u, epitheaflagallin-3-*O*-gallate; v, theaflavin; w, theaflavin-3-*O*-gallate; x, theaflavin-3′-*O*-gallate; and y, theaflavin-3,3′-di-*O*-gallate (see the Supporting Information for structures).

Hydrolysis of 4. A solution of **4** (10 mg) in H_2O (1 mL) was stirred with tannase (3 mg) at room temperature for 12 h, and the mixture was directly applied to a column of Chromatorex ODS (1 cm × 15 cm) with 0−60% MeOH (10% stepwise elution) to give gallic acid (1 mg) and **4a** (4 mg).

Acetonyl Theacitrin A (5). Brown amorphous powder, $[\alpha]_D^{26}$ 70.2° (*c* 0.1, MeOH). FAB-MS *m*/*z*: 819 $[M + H]^+$. HR-FAB-MS *m*/*z*: 819.1799 $[M + H]^+$ ($C_{40}H_{34}O_{19}$ requires 819.1773). UV λ_{max}^{MeOH} nm (log *ε*): 210 (4.82), 278 (4.13). IR ν_{max} cm^{-1}: 3397, 1709, 1615, 1519, 1470. ^{1}H NMR (500 MHz, in acetone-d_6) *δ*: 6.95 (2H, s, galloyl-2, 6), 6.57 (1H, s, B-c), 6.10 (1H, d, *J* = 2.0 Hz, A′-6), 6.08 (1H, d, *J* = 2.0 Hz, A-6), 5.97 (1H, br s, C′-3), 5.92 (1H, d, *J* = 2.3 Hz, A′-8), 5.86 (1H, d, *J* = 2.0 Hz, A-8), 5.30 (1H, br s, C-2), 4.64 (1H, d, *J* = 1.4 Hz, C-3), 4.57 (1H, br s, C′-2), 3.63 (1H, s, B-e), 3.43, 3.24 (each 1H, d, *J* = 17.6 Hz, acetone-CH_2), 3.21 (1H, d, *J* = 15.1 Hz, B′-f), 3.04 (2H, br s, C′-4), 3.01 (1H, d, *J* = 15.1 Hz, B′-f), 2.94 (1H, dd, *J* = 4.8, 16.7 Hz, C-4), 2.76 (1H, dd, *J* = 3.9, 16.7 Hz, C-4), 2.03 (3H, s, acetone-CH_3). ^{13}C NMR (125 MHz, in acetone-d_6) *δ*: 207.4 (B′-g), 207.1 (acetone-CO-), 200.7 (B-b), 187.0 (B′-l), 182.0 (B′-i), 177.8 (B-d), 166.0 (galloyl-7), 157.9 (A-7), 157.8 (A′-7), 157.7 (A-5, A′-5), 155.3 (A-8a), 155.2 (A′-8a), 145.7

Table 1. Concentration (mg/g Dried Leaf) of Polyphenols and Xanthine Derivatives in Tea Products (60% EtOH Extract, Average of Four Measurements)

	green tea		oolong tea		black tea		new fermented tea
compounds	Nagasaki (Japan)	Zhejiang (China)	Fujian (China)	Guangdong (China)	Darjeeling (India)	Uva (Sri Lanka)	MT (Japan)
caffeine	27.70 ± 0.32	42.32 ± 1.28	24.43 ± 0.53	29.11 ± 0.23	36.61 ± 0.18	32.85 ± 0.44	21.85 ± 0.45
theobromine	0.91 ± 0.02	1.78 ± 0.04	0.47 ± 0.01	0.55 ± 0.00	1.58 ± 0.06	2.02 ± 0.01	0.38 ± 0.01
gallic acid	0.14 ± 0.01	0.68 ± 0.04	[a]	1.98 ± 0.03	2.73 ± 0.02	2.22 ± 0.06	3.03 ± 0.12
theogallin	1.06 ± 0.02	10.83 ± 0.40	0.65 ± 0.02	0.95 ± 0.02	3.59 ± 0.02	3.73 ± 0.03	0.30 ± 0.03
epicatechin	11.94 ± 0.76	9.95 ± 0.63	11.95 ± 0.23	6.58 ± 0.68	4.70 ± 0.57	2.15 ± 0.10	1.34 ± 0.24
epigallocatechin (**7**)	49.84 ± 0.53	21.70 ± 0.83	47.64 ± 1.42	17.88 ± 0.13	11.56 ± 1.01	7.28 ± 0.10	1.88 ± 0.07
epicatechin-3-*O*-gallate	16.67 ± 0.15	31.08 ± 0.77	14.71 ± 0.25	13.43 ± 0.14	18.82 ± 0.65	11.39 ± 0.09	3.03 ± 0.22
epigallocatechin-3-*O*-gallate (**6**)	76.93 ± 0.94	85.53 ± 1.80	58.34 ± 1.00	42.37 ± 0.38	43.48 ± 0.84	15.56 ± 0.18	7.45 ± 0.51
catechin	6.71 ± 9.56	2.56 ± 0.28	0.75 ± 0.06	1.67 ± 0.08	1.42 ± 0.23	0.17 ± 0.01	0.39 ± 0.14
gallocatechin	4.48 ± 0.21	2.69 ± 0.10	2.93 ± 0.09	5.52 ± 0.08	2.38 ± 0.05	[a]	[a]
gallocatechin-3-*O*-gallate	4.35 ± 0.09	8.83 ± 0.36	1.80 ± 0.52	4.82 ± 0.07	3.32 ± 0.67	1.44 ± 0.02	0.72 ± 0.17
theasinensin A	[a]	1.57 ± 0.17	0.95 ± 0.06	1.56 ± 0.03	9.24 ± 0.49	8.36 ± 0.11	4.61 ± 0.16
theasinensin B (**8**)	[a]	[a]	[a]	1.30 ± 0.03	4.94 ± 0.04	7.22 ± 0.06	3.81 ± 0.16
polymeric polyphenols	8.97 ± 0.08	[b]	16.58 ± 1.11	52.31 ± 0.76	45.82 ± 3.92	59.46 ± 0.66	64.87 ± 3.51
total theaflavins	[a]	[a]	[a]	0.69 ± 0.02	3.52 ± 0.12	7.83 ± 0.11	4.92 ± 0.08
			theaflavin composition				
theaflavin				25.3%	25%	18.9%	15.0%
theaflavin-3-*O*-gallate				20.9%	22%	27.0%	31.0%
theaflavin-3′-*O*-gallate				21.9%	16%	18.2%	13.6%
theaflavin-3-3′-*O*-gallate				32.0%	36%	35.9%	40.5%

[a] Undetectable level. [b] Not determined.

(galloyl-3, 5), 139.0 (galloyl-4), 130.1 (B-c), 121.2 (galloyl-1), 111.7 (B′-j), 110.1 (galloyl-2, 6), 100.0 (A-4a), 99.5 (A′-4a), 97.9 (A′-6), 97.1 (A-6), 95.5 (A-8), 94.9 (A′-8), 84.3 (B-a), 82.9 (C′-2), 77.6 (C-2), 74.5 (B′-h), 67.2 (C-3), 64.8 (C′-3), 57.3 (B-e), 50.9 (acetone-CH_2), 48.2 (B′-k), 47.9 (B′-f), 30.0 (acetone-CH_3), 28.5 (C-4), 27.8 (C′-4).

RESULTS AND DISCUSSION

HPLC Analysis. In our previous study, the major polyphenols of MT were shown to be catechins, theasinensins, theaflavins, and polymeric polyphenols produced by enzymatic oxidation of the monomeric catechins.[6] The HPLC profile of the 60% EtOH extract is presented in Figure 1, and the concentration of the constituents is compared to those of green tea (products of Japan and China), oolong tea (two products of China), and black tea (products of India and Sri Lanka) in Table 1 (see the Supporting Information for chromatograms). The polymeric polyphenols were obtained by size-exclusion chromatography in our previous studies[6,12] and detected as a broad hump on the HPLC baseline in the usual HPLC analysis (Figure 1). The amount was estimated by a modified method described in the previous study.[6] Chemical characterization of the polymeric polyphenols was described in previous studies, including spectroscopic comparisons and in vitro model experiments that mimic tea fermentation.[6−8,10,12] Although details are still unclear, our results suggest that catechins are polymerized mainly by oxidative B ring−B ring couplings and B ring−galloyl couplings. The polyphenol composition of green tea is similar to the fresh leaves, because the leaves are steamed or roasted to inactivate enzymes involving catechin oxidation at the initial stage of the manufacture. Oolong tea is produced through a unique process including withering under the sun light and shaking in the shade. It contains lower amounts of tea catechins and higher amounts of polymeric polyphenols as compared to green tea. Black tea and MT contain theasinensins and theaflavins together with a large amount of polymeric polyphenols. These characteristic compounds are produced by mixing tea catechins with the oxidation enzymes on crushing and twisting of the fresh leaves. The concentration and composition of polyphenols in tea products depend not only on differences in processing but also tea plant cultivars used for the production. Cultivars of tea plants belong to two major varieties, *C. sinensis* var. *sinensis* and var. *assamica*. The latter type is mainly used for black tea production and contains larger amounts of tea catechins as compared to those of the former. The MT was produced from cultivar Yabukita, belonging to the *sinensis* type, and its catechin and caffeine concentrations tend to be lower than those of other tea products.

Isolation and Identification of Polyphenols. Previously, we reported separation of the major polyphenol components, such as theasinensins, theaflavins, and polymeric polyphenols,[6] which are probably important in the biological activities,[4,5] and we then investigated the minor constituents to find polyphenols characteristic to this tea product. Further separation of the remaining fractions after separation of the major compounds[6] afforded gallic acid, 1-*O*-galloyl-*β*-D-glucose,[13] 1,6-di-*O*-galloyl-*β*-D-glucose,[14] 1,4,6-tri-*O*-galloylglucose,[15] strictinin,[15] procyanidin B-2,[16] procyanidin B-4,[16] (−)-epigallocatechin-3-*O*-gallate (**6**), (−)-epigallocatechin (**7**), (−)-epicatechin-3-*O*-gallate, (−)-epicatechin, (+)-catechin, (+)-gallocatechin, theasinensin B (**8**),[11,17] theasinensin C,[11,17] theaflavonin,[18] 2,3,5,7-tetrahydroxychroman-3-*O*-gallate,[10] myricetin 3-*O*-glucoside, quercetin 3-*O*-galactoside, quercetin 3-*O*-glucoside, theacitrinin A (**1**),[19] and compound

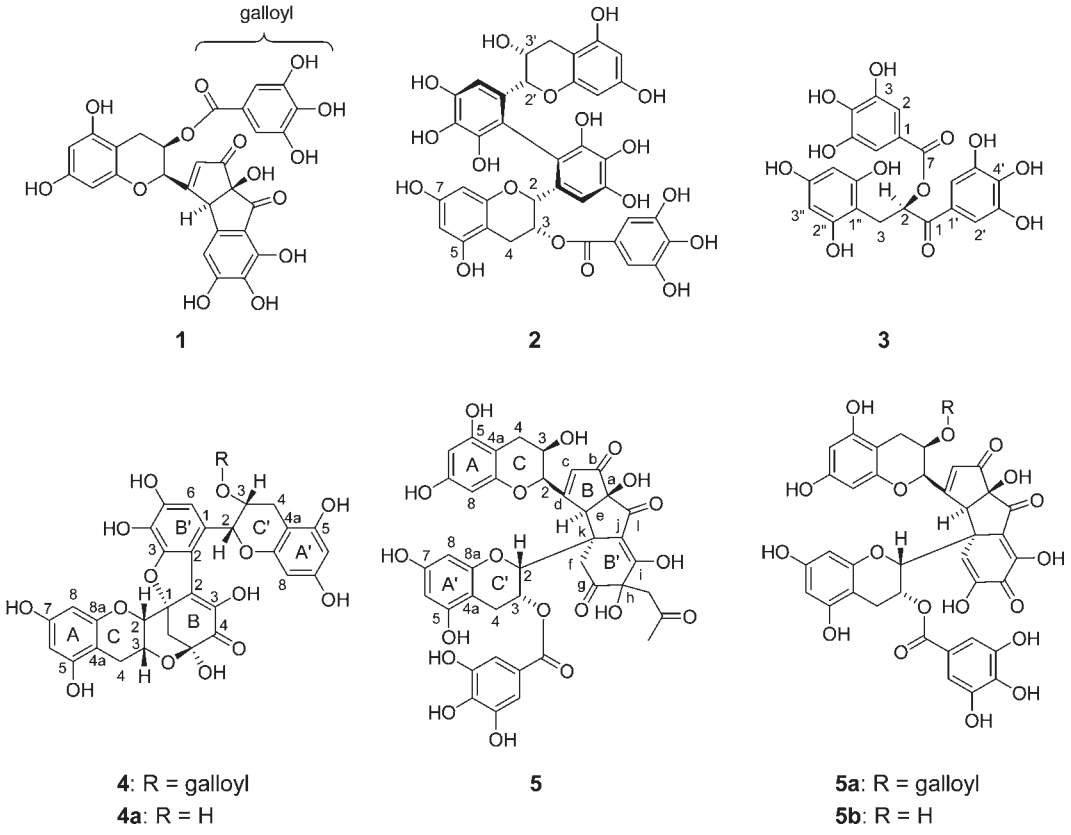


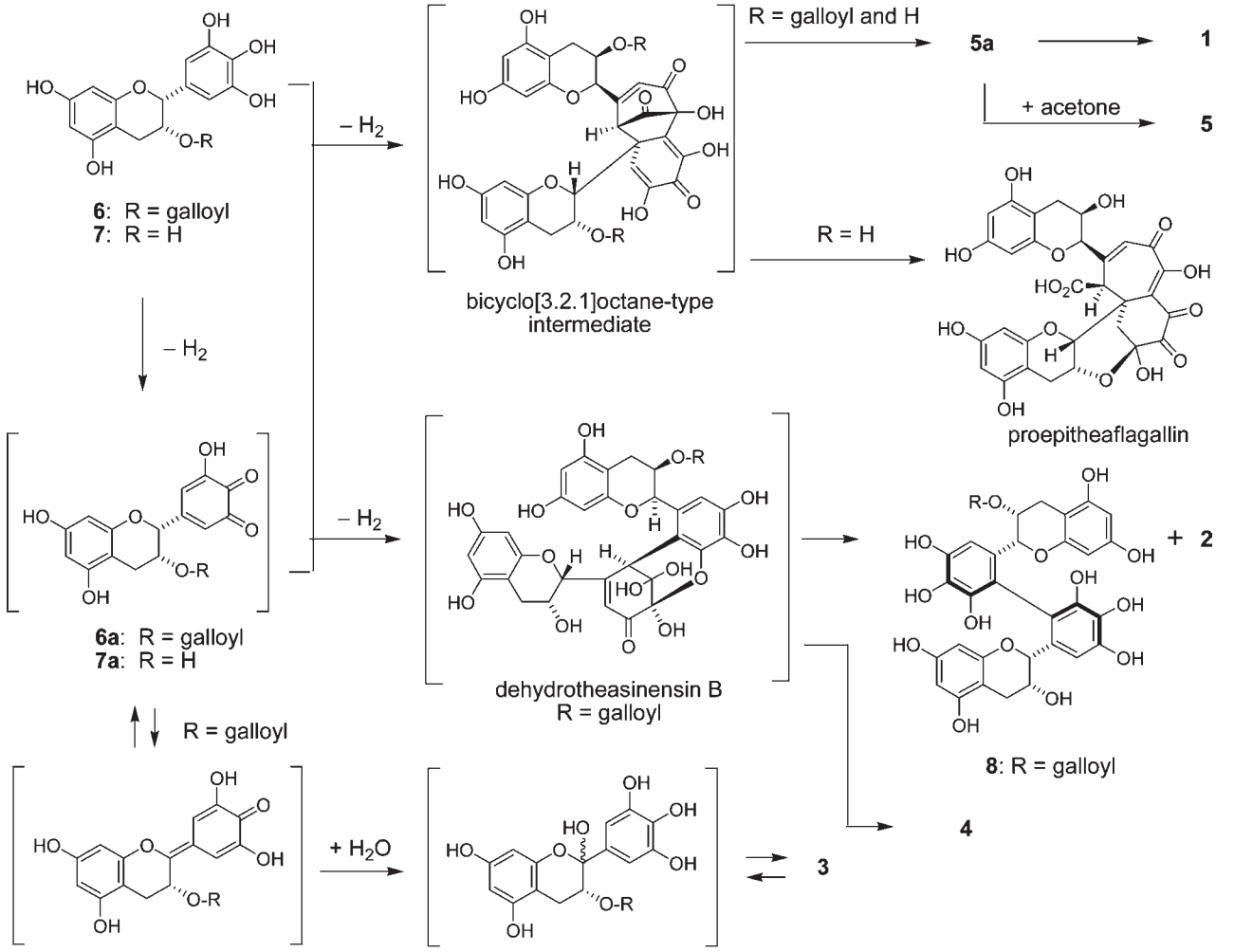
Figure 2. Structures of compounds **1**−**5**, **4a**, **5a**, and **5b**.

2[11] (Figure 2). These known compounds were identified by comparison of spectroscopic data with those of authentic samples or data described in the literature (see the Supporting Information for structures). Among the known compounds, **1** was originally identified as an in vitro degradation product of theacitrin C (**5a**),[19,20] and this is the first time it has been isolated from a commercial fermented tea product (MT). The configuration of the tricyclic B ring structures of **1** and **5a** was deduced to be as shown Figure 2 based on an assumption that **5a** and proepitheaflagallin are produced from the same bicyclo[3.2.1] octane type intermediate (Scheme 1).[19,21] The configuration of proepitheaflagallin was established by chemical and spectroscopic methods in our previous study.[22] Compound **2** is an atrop isomer of theasinensin B (**8**), and the structure was confirmed by hydrolysis with tannase yielding theasinensin E with the *S*-biphenyl bond. This compound was originally obtained by oxidation of a mixture of (−)-epigallocatechin-3-*O*-gallate (**6**) and (−)-epigallocatechin (**7**) with $K_3Fe(CN)_6$ under weakly alkaline conditions.[11] In this study, compound **2** was first isolated from commercial tea products and named theasinensin H, because to date, seven analogues of B−B′ linked catechin dimers have been isolated from tea products and named theasinensins A−G, respectively.[11] On the basis of the evidence shown by in vitro enzymatic oxidation,[10] **2** and **8** were deduced to be produced by degradation of dehydrotheasinensin B (Scheme 1).

Compound **3** was isolated as a tan, amorphous powder, and the FAB-MS exhibited a $[M + H]^+$ peak at *m*/*z* 475, indicating that the molecular weight of **3** is 16 mass units larger than that of **6**. The ^1H and ^{13}C NMR spectra of **3** were related to those of **6** and revealed the presence of a galloyl group (δ_H 7.18, 2H, s), a pyrogallol type B ring (δ_H 7.38, 2H, s), and a phloroglucinol-A ring (δ_H 6.01, 2H, s). In addition, mutually coupled proton signals due to oxygen-bearing methine (δ_H 6.17, dd, 3.4 and 10.3 Hz) and benzylic methylene [δ_H 3.14 (dd, 3.4 and 14.0 Hz) and 3.25 (dd, 10.3 and 14.0 Hz)] were observed. However, the C ring H-2 signal of **6** disappeared in the spectrum of **3**, and a conjugated carbonyl carbon signal appeared at δ_C 195.6 (C-1) instead of the C-2 methine carbon of **6** in the ^{13}C NMR spectrum. A large low-field shift of the B ring pyrogallol proton (δ_H 7.38) as compared to that of **6** (δ_H 6.63) indicates that the carbonyl group was connected to the B ring. The HMBC spectroscopic examination (Figure 3) establishes the arrangement of the partial structures. The configuration of the methine carbon was deduced to be the same as that of **6** from the following biogenetic consideration. During production of MT, enzymatic oxidation of **6** affords an *ortho*-quinone **6a**,[6,7] and subsequent tautomerization and hydration of **6a** yields **3**. (Scheme 1). Accordingly, **3** was concluded to be (2*R*)-2-hydroxy-3-(2,4,6-trihydroxyphenyl)-1-(3,4,5-trihydroxyphenyl)-1-propanone 2-*O*-gallate.

Compound **4** is a yellow pigment and showed an UV absorption maximum at 384 nm. The ^1H and ^{13}C NMR spectra revealed presence of two sets of flavan-3-ol A and C rings along with a galloyl ester moiety (δ_H 6.79, 2H, s). The dimeric nature was also indicated by MALDI-TOF-MS (*m*/*z* 783, $[M + Na]^+$). The ^{13}C NMR spectrum exhibited signals arising from a pyrogallol type aromatic ring (δ 152.2, 151.0, 130.8, 129.4, 112.4, and 110.2), a carbonyl (δ 189.6, B-4), a fully substituted double bond (δ 139.7, B-3; 134.3, B-2), an acetal (δ 94.7, B-5), an oxygenated quaternary carbon (δ 89.6, B-1), and a methylene (δ 40.7, B-6).

Scheme 1. Oxidation of 6 and 7 and Production of 1−5



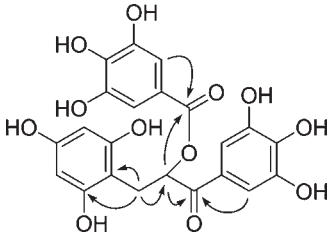


Figure 3. Selected HMBC correlations for **3**.

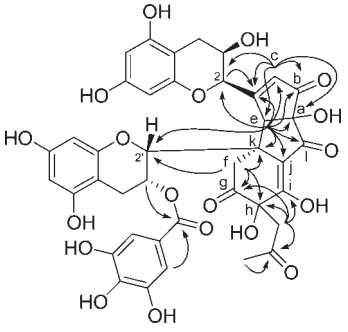


Figure 4. Selected HMBC correlations of **5**.

These signals were closely related to the B ring signals of dehydrotheasinensin E (**4a**), which were originally obtained as an in vitro enzymatic oxidation product of epigallocatechin (**7**).[7] In addition, enzymatic hydrolysis of **4** with tannase afforded **4a** and gallic acid. The location of the galloyl group was determined to be at the C′-3 hydroxyl group due to the large low field shift of H−C′-3 (δ_H 5.94, br s) as compared to that of **4a** (δ_H 4.26). Thus, compound **4** was characterized to be the 3′-*O*-galloyl ester of **4a** and named dehydrotheasinensin H, because **4** and **4a** are equivalent to the oxidized form of theasinensins H (**2**) and E, respectively. Compound **4** is probably produced by isomerization and dehydration of dehydrotheasinensin B, an isomer with differing distortion of the C−C bond between B and B′ rings (Scheme 1).

The ^{13}C NMR spectrum of compound **5** was partially related to that of theacitrin A (**5b**), which is an unstable yellow pigment isolated from black tea.[20] Signals attributable to a cyclopentanone ring (δ_C 84.3, C-a; 200.7, C-b; 130.1, C-c; 177.8, C-d; and 57.3, C-e) were similar to those of **5b**, as well as the signals of two sets of flavan-3-ol A and C rings and a galloyl group. Examination of the HSQC spectroscopy indicated that the remaining 10

carbon signals were assigned to three carbonyls (δ_C 207.4, C-g; 207.1, acetone CO; and 187.0, C-l), an oxygenated double bond (δ_C 182.0, C-i; 111.7, C-j), an oxygenated quaternary carbon (δ_C 74.5, C-h), a quaternary carbon (δ_C 48.2, C-k), two methylenes (δ_C 50.9, acetone-CH_2; 47.9, C-f), and a methyl carbon (δ_C 30.0, acetone-CH_3). In the HMBC spectrum, the methine proton H-e (δ 3.63, s) of the above-mentioned cyclopentanone ring was correlated with C-j, C-k, and C-l in addition to two flavan-3-ol C ring C-2 carbons, confirming the close structural relationship between **5** and **5b**. The most notable difference between **5** and **5b** was the appearance of the acetone moiety, which was indicated by HMBC correlations of a carbonyl carbon (δ_C 207.1) with methyl (δ_H 2.03, s) and methylene protons (δ_H 3.43, 3.24, each d, J = 17.6 Hz) (Figure 4). Furthermore, one of the flavan-3-ol C-2 carbons (δ_C 82.9, C-2′) was correlated with the methylene protons at δ_H 3.21 and 3.01 (each 1H, d, J = 15.1 Hz, H-f), which also showed correlation peaks with the carbons of C-k, C-g, and C-h. The methylene protons of the acetone moiety were correlated with the carbonyl (C-g), an oxygenated sp^2 carbon (C-i) and a quaternary sp^3 carbon (C-h), indicating that the acetone moiety was attached to the C-h. On the basis of this spectroscopic evidence, compound **5** was determined to be an acetone adduct of theacitrin A (**5b**) and named acetonyl theacitrin A. Although **5** is apparently an artifact produced during extraction with aqueous acetone, the isolation of this compound indicates that theacitrin type pigments were present in the MT. Theacitrins, such as **5a** and **5b**, are unstable in aqueous solution and give degradation products including theacitrinin type compounds.[19] Because **5** is relatively stable as compared to **5b**, production of acetone adducts may have some significance, because similar reactions of dehydroellagitannins with acetone were applied to preparation of stable derivatives useful to structure determination and selective HPLC detection.[23]

The production mechanism proposed for **1**−**4** isolated in this study (Scheme 1) suggests that these compounds are probably common in black tea. However, our results include some significant findings concerning catechin oxidation. The most important is that isolation of compounds **1**, **2**, and **4** from a commercial fermented tea product provides evidence that the reactions hitherto identified only in in vitro oxidation experiments actually occur in the tea leaves during tea fermentation. In addition, isolation of **5** shows the presence of theacitrins in MT, and production of **3** demonstrates the occurrence of tautomerization and hydration of quinone intermediates of tea catechins. Our previous studies using in vitro tea fermentation model experiments[8,10,19,21,22] suggest that production of well-known black tea polyphenols, such as theaflavins and theasinensins, are accompanied by a large number of uncharacterized minor compounds. The minor compounds are probably related to so-called "thearubigins", which are uncharacterized black tea polyphenols and account for the major portion of polyphenols in black tea infusions.[2] Therefore, studies on minor catechin oxidation products in various type of fermented tea are important to clarify the chemical composition of black tea, which accounts for almost 80% of world tea production.

■ ASSOCIATED CONTENT

Supporting Information. HPLC profiles, chemical structures, and NMR and MS spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

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