Oligomerization Mechanisms of Tea Catechins Involved in the Production of Black Tea Thearubigins

Keigo Hashiguchi,[†] Sena Teramoto,[†] Kohei Katayama,[‡] Yosuke Matsuo,[†] Yoshinori Saito,[†] Takashi Tanaka[†,*]

[†] Graduate School of Biomedical Sciences, Nagasaki University, 1-14 Bunkyo-machi, Nagasaki 852-8521, Japan

[‡] School of Pharmaceutical Sciences, Nagasaki University, 1-14 Bunkyo-machi, Nagasaki 852-8521, Japan

[*]Corresponding author: Tel: 81 (0)95 819 2432; Email: t-tanaka@nagasaki-u.ac.jp

ABSTRACT: Thearubigins (TRs) are chemically ill-defined black tea pigments composed of numerous catechin oxidation products. TRs contain oligomeric components; however, the oligomerization mechanisms are poorly understood. The comparison of the ^{13}C nuclear magnetic resonance (NMR) spectra of the TRs with different molecular sizes suggested the participation of A-ring methine carbons in the oligomerization. Crushing fresh tea leaves with phloroglucinol, a mimic of the catechin A-rings, yielded the phloroglucinol adducts of the B-ring quinones of pyrogallol-type catechins and dehydrotheasinensins, indicating that the intermolecular oxidative couplings between pyrogallol-type B-rings and A-rings are involved in the oligomerization. This is supported by the comparison of the ^{13}C NMR spectra of the oligomers generated from the dehydrotheasinensins and epicatechin. Furthermore, the presence of the quinones or related structures in the catechin oligomers is shown by condensation with 1,2-phenylenediamine. The pyrogallol-type catechins account for approximately 70% of tea catechins; therefore, the B–A ring couplings of the pyrogallol-type catechins are important in the catechin oligomerization involved in TR production.

KEYWORDS: *black tea, thearubigins*, *catechin, dehydrotheasinensin, quinone, oxidation*

INTRODUCTION

Two major tea products, green tea and black tea, are produced from the same tea plant (*Camellia sinensis*), and the latter accounts for approximately 80% of the world's tea production.[1] Tea polyphenols in the fresh leaves mainly comprise (−)-epigallocatechin (**1**), (−)-epigallocatechin-3-*O*-gallate (**2**), (−)-epicatechin (**3**), and (−)-epicatechin-3-*O*-gallate (**4**) (**Figure 1**). In green tea production, tea leaves are heated immediately after harvesting to deactivate leaf enzymes; therefore, the polyphenol composition is similar to that of fresh leaves. In contrast, in black tea production, the leaves are withered and mechanically crushed to mix the polyphenols with leaf enzymes, and enzymatic reactions produce a complex mixture of catechin oxidation products, comprising black tea polyphenols.[2] This process is the so-called "aeration" (formerly called tea-fermentation). The high-performance liquid chromatography (HPLC) of a commercial black tea shows sharp peaks for theasinensins (**5**–**7**), theacitrins (**8**, **9**), and theaflavins (**10**–**13**) (**Figure 1**). These conspicuous catechin dimers are representative black tea polyphenols and chemically well characterized.[2] However, the major components of the black tea polyphenols are a mixture of oligomeric polyphenols, thearubigins (TRs),[3-5] which is detected as a Gaussian-shape broad hump on the HPLC baseline.[2,5-7] The TRs account for 60%–70% of the dry substance of black tea infusion,[1,4] and the biological activities of the TRs or fractions containing TRs are reported.[1,8-10] However, with our current knowledge about the chemical structures and production mechanisms of the TRs, the chemical definition of the TRs has not been established. In order to elucidate the production mechanisms, catechin oxidation under various conditions have so far been investigated in detail.[2] Major catechin dimers (**5**–**13**) are produced by oxidative B–B ring couplings, and their

production mechanisms have been well established.[2,11-15] Additionally, identification of some minor trimeric and tetrameric products derived from the dimers suggest that oligomerization mechanisms include oxidative couplings between galloyl groups and catechol- (**Figure 2B**)[16-21] or pyrogallol-type B-rings (**Figure 2C**).[22,23] The reactions of the benzotropolone rings of **10**–**13** or their oxidation products, such as theanaphthoquinone (**Figure S2**),[24] may contribute to the oligomerization (**Figures 2D–G**).[19,25,26] However, the in vitro enzymatic oxidation of pure catechins (**Figure S1**) and combinations of catechins (**Figure S2**) indicates that the dimeric products are considerably stable in each reaction mixture. Thus, mechanisms that directly produce oligomeric products from monomeric catechins possibly exist in TR production. The oxidation of **3** alone with polyphenol oxidase affords B–A ring coupling products (**Figure 2A**).[2,27,28] In the reaction, dimeric products are only detected in trace amounts, and oligomeric products are dominantly accumulated (**Figure S1**). This is because the B–A ring coupling products retain unreacted A- and B-rings, which undergo further couplings. However, during the aeration of tea leaves, the catechol-type B rings mainly react with pyrogallol-type B rings to produce theaflavins; therefore, B-A ring couplings of the catechol-type catechins are probably not important in TR production.[2] If similar sequential B–A ring couplings occur in the oxidation of the pyrogallol-type catechins, the mechanisms are possibly the major route of the TR production occurring in a background of conspicuous dimerization reactions. This is because the pyrogallol-type catechins (**1** and **2**) account for approximately 70% of the tea catechins.[29] Based on this assumption, this paper describes the results of several model aeration experiments and the chemical investigation of the oxidation of the pyrogallol-type catechins. This study mainly composed of 4 experiments: first, time course of polyphenol compositions

during aeration of tea leaves were examined. Second, TRs were analyzed by size-exclusion chromatography and spectroscopic analysis. Third, aeration of tea leaves with phloroglucinol as a mimic of catechin A ring was performed, and finally, decomposition of a quinone dimer of **2** was investigated in detail.

MATERIALS AND METHODS

Materials. Green tea leaves (*Camellia sinensis* var. *sinensis*) were supplied by the Nagasaki Agriculture and Forestry Technical Development Center, Higashisonogi Tea Research Station. Japanese pear fruits were purchased in a local market in Nagasaki city. Tea catechins **1**–**4** were isolated from commercial green tea according to a reported method.[30] Epigallocatechin-3-*O*-gallate (**2**) was obtained from Taiyo Kagaku Co. Ltd. (Yokkaichi, Japan). All chemicals and reagents were of analytical grade. No.2 Filter paper was purchased from Toyo Roshi Kaisha, Ltd. (Tokyo, Japan). Commercial black tea products were purchased from LUPICIA Co., Ltd. (Tokyo, Japan).

Analytical procedures. Ultraviolet–visible (UV–vis) spectra were obtained using a Jasco V-560 UV–Vis spectrophotometer, and infrared (IR) spectra were measured on a JASCO FT/IR 410 spectrophotometer (Jasco Co., Tokyo, Japan). Optical rotations were measured using a Jasco DIP-370 digital polarimeter. Electronic circular dichroism (ECD) spectra were measured using a JASCO J-725N spectrophotometer. ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectra were recorded in acetone-d_6 at 27°C or dimethyl sulfoxide (DMSO)-d_6 at 70°C using a Varian NMR System 500PS SN spectrometer (Varian, Palo Alto, CA) operating at 500 MHz for ^{1}H and 126 MHz for ^{13}C. ^{1}H–^{1}H correlation spectroscopy, rotating frame Overhauser effect spectroscopy (ROESY), heteronuclear single quantum correlation, and heteronuclear multiple bond

correlation (HMBC) experiments were performed using standard Varian pulse sequences. Fast atom bombardment mass spectra (FABMS) were recorded on a JEOL JMS-700N spectrometer (JEOL Ltd., Tokyo, Japan), and glycerol or *m*-nitrobenzyl alcohol was used as the matrix. Column chromatography was performed using Diaion HP20SS (75–150 μm) (Mitsubishi Chemical Co. Tokyo, Japan), Sephadex LH-20 (25–100 μm) (GE Healthcare Bio-Science AB, Uppsala), and Chromatorex ODS (Fuji Silysia Chemical Ltd., Kasugai, Japan). Thin-layer chromatography (TLC) was performed on 0.2 mm precoated Kieselgel 60 F_{254} plates (0.2-mm thick, Merck KGaA, Darmstadt, Germany) with toluene-ethyl formate-formic acid (1:7:1, v/v). Spots were detected by illumination under a short UV wavelength (254 nm), followed by spraying with 2% ethanolic $FeCl_3$. Analytical HPLC was performed on a 250 × 4.6 mm i.d. Cosmosil 5C_{18}-AR II column (Nacalai Tesque Inc., Kyoto, Japan) with the gradient elution of CH_3CN in 50 mM H_3PO_4 from 4% to 30% in 39 min and 30% to 75% in 15 min at a flow rate of 0.8 mL/min at 35 °C. Further, the detection was performed using a Jasco MD-2018Plus photodiode array detector. Size-exclusion chromatography was performed on a TSK-gel α3000 column (300 × 7.8 mm i.d.) at 40 °C with dimethylformamide containing 10 mM LiCl as the elution solvent at a flow rate of 0.8 mL/min.[9] Polystyrenes (molecular weights of 4000, 25000, 50000, and 170000, Nacalai Tesque Inc., Kyoto, Japan) were used as standards, and the molecular weight distribution was calculated using the ChromNAV GPC/SEC calculation program (Jasco Co., Tokyo, Japan). Sanguiin H-2 (MW 1870) and lambertianin C (MW 2804) were isolated from *Rubus parvifolius* L. as phenolic standards.[31,32]

Model aeration using lyophilized fresh tea leaves. Fresh tea leaves collected in May were withered at room temperature for 18 h and lyophilized using a freeze dryer

(FDU-1200, Tokyo Rikakikai Co., Ltd., Tokyo, Japan). The dried leaves were pulverized using a Waring blender and passed through a sieve (0.85 mm) to remove the petioles, and the powder (each 0.50 g) was dispensed onto 10 plastic trays (5 × 5 cm). Aeration was carried out by mixing H_2O (2.0 mL) with the leaf powder at room temperature and occasionally stirring using a micro spatula. After 10, 20, 40, 60, 90, 120, 150, 180, 210, and 240 min, respectively, one tray was picked up, wrapped with a plastic wrapping sheet, and heated in a microwave oven (500 W, 15 s). The heated tea leaf powder in the tray was lyophilized, and a portion (50 mg) was extracted with 60% EtOH (5.0 mL) in a screw-capped vial (10 mL) at 70°C for 1 h. The extract was filtered through a membrane filter (0.45 μm, Sartorius Stedim Biotech GmbH, Goettiingen, Germany) and analyzed by HPLC (10 μL) (**Figure S3**).

TR fractions from aerated tea leaves. The lyophilized tea leaf powder was dispensed onto four plastic trays (each 1.0 g) and mixed with H_2O (4 mL). After stirring for 60, 120, and 180 min, the samples were separately lyophilized as described above. Each tea leaf powder (0.70 g) was extracted twice with 60% MeOH (50 mL) at 60°C for 1 h. The extract was concentrated and subjected to column chromatography using the Sephadex LH-20 column (2 cm i.d. × 7 cm). The column was first washed with 60% MeOH (50 mL) to eliminate caffeine and flavonoid glycosides. The subsequent elution of the column with 70% acetone (50 mL) afforded a fraction of catechin oxidation products. An aqueous solution (approximately 10 mL) of the fraction was partitioned three times with a mixture of EtOAc–hexane (4:1, v/v, 10 mL) in a centrifuge tube (50 mL),[33] and the aqueous layer containing crude TRs was concentrated and lyophilized (46–49 mg).

Separation of TRs from black tea. Commercial black tea (product of Kenya, 20 g) was extracted twice with 60% acetone. After the removal of acetone using a rotary

evaporator, precipitates were removed by filtration (No. 2 filter paper). Thereafter, the filtrate was fractionated using a Diaion HP20SS (3 cm i.d. × 18 cm) with 0%–100% of MeOH (10% stepwise, each 100 mL) to afford 6 fractions: fr. 1 (sugars), fr. 2 (gallic acid, theogallin, **5**, **6**, **8**, and TRs), fr. 3 (**6**, **8**, **9**, caffeine, and TRs), fr. 4 (**2**, **7**, caffeine, and TRs), fr. 5 (**4**, caffeine, and TRs), and fr. 6 (**10**–**13** and TRs). The fr. 2 and 3 were combined and separated by size-exclusion chromatography using a Sephadex LH-20 (4 cm i.d. × 45 cm) eluted with a mixture of 7 M urea containing conc. HCl (5 mL/L) and acetone (2:3, v/v).[34] The fractions containing only oligomeric polyphenols, which were detected at the origin on a silica gel TLC plate, were concentrated to remove acetone. The resulting aqueous solution was applied to Diaion HP20SS column chromatography (2 cm i.d. × 15 cm) with 0%–100% MeOH (10% stepwise, each 50 mL) to afford **TR$_{15–35\ min}$** (200.8 mg), which was detected as a broad hump on HPLC baseline between t_R 15 min and 35 min (Figure 5). Using the same procedure, **TR$_{25–50\ min}$** (340.8 mg) was obtained from fr. 5.

Aeration of fresh tea leaveswith phloroglucinol. Fresh tea leaves (700 g) collected in June were homogenized with an aqueous solution of phloroglucinol (35 g in 450 mL of H_2O) in a Waring blender. The crushed leaves were spread on a plastic tray (30 cm × 45 cm) in a thermostatic chamber (the leaf temperature was approximately 30°C) and occasionally stirred and kneaded using a spatula. After 3 h, the leaves were heated at 80°C for 3 h in a drying chamber and extracted with 70% acetone (3 L). After filtration, the debris was further extracted twice with the same solvent (1 L). The extracts were combined and concentrated using a rotary evaporator. The precipitates formed in the resulting aqueous solution were removed by filtration, the filtrate (approximately 2 L) was acidified (approximately pH 3) using trifluoroacetic acid (TFA), and the insoluble

aggregates were dissolved by adding MeOH (300 mL). The mixture was applied to a column of the Sephadex LH-20 (7 cm i.d. × 30 cm) and washed with 20% MeOH. Further, the elution of the column with 40%–100% MeOH (10% stepwise, each 500 mL), MeOH–acetone–H_2O (8:1:1, v/v/v, 500 mL), and 60% acetone (2 L) afforded 10 fractions (fr.): fr. 1 (18.4 g), fr. 2 (6.10 g), fr. 3 (4.92 g), fr. 4 (2.95 g), fr. 5 (4.42 g), fr. 6 (6.23 g), ft. 7 (2.61 g), fr. 8 (4.29 g), fr. 9 (3.81 g), and fr. 10 (5.35 g). Fr. 1 and 2 were mainly composed of phloroglucinol, caffeine, flavonol glycosides, gallic acid, **1**, and **3**. Fr. 3 was separated by Diaion HP20 column chromatography (4 cm i.d. × 25 cm, 0%–80% MeOH, 5% stepwise, each 200 mL) to afford **14** (638 mg). Fr. 4 was subjected to Diaion HP20 chromatography (4 cm i.d. × 25 cm, 0%–80% MeOH) and Chromatorex ODS (3 cm i.d. × 25 cm, 0%–30% MeOH, 5% stepwise, each 100 mL) to afford **16** (154.7 mg). Fr. 5 was applied to the Diaion HP20 column (4 cm i.d. × 25 cm, 0%–80% MeOH) to yield **15** (626 mg), along with a crude sample of **15** (660 mg). Fr. 6, mainly composed of **2** and **4**, was separated using the Diaion HP20 column (4 cm i.d. × 25 cm, 0%–80% MeOH, 5% stepwise, each 100 mL) to afford 7 subfractions. Fr. 6-3 (2.07 g) was further purified using Chromatorex ODS (4 cm i.d. × 25 cm, 0%–50% MeOH, 5% stepwise, each 200 mL) and Sepahdex LH-20 (3 cm i.d. × 20 cm, 90% EtOH) to yield **18** (100.0 mg). Fr. 7 was first fractionated by size-exclusion chromatography using Sephadex LH-20 (4 cm i.d. × 45 cm) to remove oligomers. The fractions containing polyphenols with relatively low molecular weights were separated by Diaion HP20 column chromatography (4 cm i.d. × 45 cm, 0%–100% MeOH, 5% stepwise each 200 mL) to afford **17** (749.9 mg). Fr. 8 and fr. 9 were separated in a similar manner described for fr. 7, **7** (354 mg) and **10** (300 mg) were isolated from fr. 8, and **19** (548.2 mg) and a mixture of theaflavin galloyl esters (795.6 mg) were obtained

189 from fr. 9. Physicochemical data of **14** – **19** are shown in supporting information.

190 **Preparation of 14 and 16 from 1.** Japanese pear fruit (100 g) was homogenized with
191 H_2O (50 mL) and filtered through four layers of gauze. The filtrate (100 mL) was mixed
192 with an aqueous solution (100 mL) containing **1** (700 mg) and phloroglucinol (5 g) and
193 stirred for 2 h. The mixture was mixed with EtOH (300 mL) and filtered through No. 2
194 paper under reduced pressure. The filtrate was concentrated to remove EtOH, and the
195 aqueous solution was separated by Sephadex LH-20 (3 cm i.d. × 20 cm, 20%–80%
196 MeOH, 10% stepwise, each 100 mL) and Chromatorex ODS (3 cm i.d. × 25 cm,
197 0%–40% MeOH, 5% stepwise, each 100 mL) to afford **14** (256 mg) and **16** (48.2 mg).

198 **Preparation of dehydrotheasinensins A (19a) and C (16a).** Dehydrotheasinensin A
199 (**19a**) was prepared by the method originally developed for the preparation of **7**.[35]
200 Briefly, anhydrous $CuCl_2$ (10.0 g) was added to a solution of **2** (18.6 g) in H_2O (1.0 L)
201 at 40°C. The solution was vigorously stirred using a mechanical mixer for 3 h. The
202 reaction mixture was directly applied to a Diaion HP20 column (7 cm i.d. × 25 cm), and
203 the column was washed with 0.2% TFA in H_2O (1.5 L). The elution of the column with
204 5%–15% CH_3CN in 0.2% TFA (5% stepwise, each 500 mL) afforded **19a** (5.0 g). The
205 further elution of the column with 15%–20% CH_3CN afforded the recovery of **2** (7.8 g).
206 Dehydrotheasinensin C (**16a**) (2.62 g) was prepared from **1** (5.0 g) using a similar
207 procedure.

208 **Degradation of dehydrotheasinensin A (19a).** Small scale: eight vials (1 mL)
209 containing solutions of **19a** (1 mg/110 μL pH 6 citrate-phosphate buffer) were prepared,
210 and one was mixed with 2% TFA in CH_3CN (110 μL). The remaining 7 vials were
211 heated at 60°C for 1, 2, 5, 10, 15, 20, and 30 min and cooled in an ice bath. Each
212 solution was mixed with 2% TFA in CH_3CN (110 μL) and analyzed by HPLC. Large

scale: A solution (100 mL) of **19a** (1.0 g) in citrate-phosphate buffer (pH 6) was heated at 80°C for 10 min. After cooling in an ice bath, the reaction mixture was acidified with TFA to pH 3. Thereafter, Diaion HP20 column chromatography (3 cm i.d. × 25 cm) was performed with 0%–100% MeOH (5% stepwise, each 100 mL) to afford 4 fractions: fr. 1 (gallic acid, 62.4 mg), fr. 2 (301 mg), fr. 3 (**7** and **7a**, 280 mg), and fr. 4 (447 mg). Fr. 2 was separated by Sephadex LH-20 column chromatography (2 cm i.d. × 24 cm) with 0.2% TFA in 0%–100% MeOH (10% stepwise, each 50 mL) to yield pigment **21** (33.9 mg) and **7** (100 mg). Fr. 4 was subjected to size-exclusion chromatography using Sephadex LH-20 (2 cm i.d. × 45 cm). The fraction containing oligomers was concentrated and pathed through Diaion HP20 (2 cm i.d. × 18 cm) with 0%–100% MeOH to afford **TR$_{19a}$** (66.6 mg). The heating of a mixture of **19a** (1.0 g) and **3** (1.0 g) in a pH 6 buffer solution (100 mL) at 80°C for 5 min and separation as described above afforded **TR$_{19a,3}$** (143.6 mg).

*Pigment **21**:* Reddish brown amorphous powder. $[\alpha]_D^{23}$ − 884 (*c* 0.002, MeOH). IR ν_{max} cm^{-1}: 3740, 1679, 1649, 1617, 1529, 1453. 1303, 1199, 1129. UV (MeOH) λ_{max} (log ε): 513 (2.71), 399 (2.60), 275 (2.44), 207 (2.32). FABMS (positive) *m/z*: 713 M^+, HRFABMS *m/z*: 713.1143 M^+ (calcd. for $C_{36}H_{25}O_{16}^+$: 713.1143). ^{1}H and ^{13}C NMR data (in DMSO-d_6 + TFA-*d,* at 70°C): see **Table S3**.

Degradation of dehydrotheasinensin C (16a). A solution of **16a** (500 mg) in a citrate-phosphate buffer (pH 6, 50 mL) was heated at 80°C for 30 min. The reaction mixture was acidified to pH 3 with TFA, and Sephadex LH-20 column chromatography (3 cm i.d. × 8 cm) was performed. After washing the column with H_2O (200 mL), the polyphenols were eluted out with 70% acetone and separated by size-exclusion chromatography using Sephadex LH-20 (2 cm i.d. × 55 cm). The fraction containing

oligomers was applied to Diaion HP20 (2 cm i.d. × 15 cm) with 0%–80% MeOH to afford **TR$_{16a}$** (145.3 mg). The fraction containing dimeric products was separated by Sephadex LH-20 column chromatography (2 cm i.d. × 12 cm) with 0%–100% MeOH (20% stepwise, each 50 mL) to afford **5** (94.6 mg) and its atropisomer, theasinensin E (**5a**) (135.2 mg). The **TR$_{16a}$** (50 mg) and 1,2-phenylenediamine (20 mg) were dissolved in 50% CH_3CN (5 mL) containing TFA (100 μL) and warmed at 45°C for 1 h. After the removal of CH_3CN by evaporation, the aqueous solution was separated by Sephadex LH-20 column chromatography (2 cm i.d. × 12 cm) with 0%–100% MeOH (20% stepwise, each 20 mL), and the products were eluted out with 70% acetone. The oligomers were further purified by Chromatorex ODS (1 cm i.d. × 10 cm) with 20%–70% CH_3CN (10% stepwise, each 10 mL) to afford **TR$_{16aPhen}$** (24.5 mg).

Treatment of aerated tea leaves with 1,2-phenylenediamine. Fresh tea leaves (94 g) were withered at room temperature for 18 h. The leaves were homogenized with H_2O (160 mL) in a Warring blender and spread on a plastic tray (21 × 30 cm) in a thermostatic chamber (the leaf temperature was approximately 30°C). After 4 h, a portion (30 g, sample A) of the aerated tea leaves (total 130 g) was extracted with 60% EtOH (200 mL) for 8 h at room temperature. Another portion (30 g, sample B) was extracted with 60% EtOH (200 mL) containing 1,2-phenylenediamine (0.5 g) and TFA (1 mL) at room temperature for 8 h. Another portion (30 g, sample C) was heated in a microwave oven (500 W, 1 min) and extracted with 60% EtOH (200 mL) at room temperature for 8 h. To the extract of sample C (1 mL), 1,2-phenylenediamine (2 mg) and TFA (20 μL) were added and the solution was heated at 60°C for 30 min (sample D). Samples A–D were analyzed by HPLC.

RESULTS AND DISCUSSION

Aeration using lyophilized fresh tea leaves. Lyophilized fresh tea leaves retain enzyme activity, and the simple mixing of H_2O starts the enzymatic reactions and results in aeration.[26] This method enables small-scale experiments on a laboratory bench to monitor the catechin oxidation in tea leaves under various conditions. In this study, the wet leaf powder was heated in a microwave oven at designated time points for aeration. There are two purposes of the heating: one is to deactivate the oxidation enzyme, and the other is to convert unstable theasinensin precursors (**16a**–**19a**) to stable theasinensins (**16**–**19**).[11,12] The change in polyphenol compositions based on the relative values of each peak area/caffeine at 235 nm is shown in **Figure 3**. Here, caffeine in the original tea leaves was used as an internal standard. Pyrogallol-type catechins **1** and **2** decreased significantly faster than catechol-type catechins **3** and **4** (**Figure 3A**). The slow decrease in **3** was explained by the coupled-oxidation mechanism, that is, the enzyme preferentially oxidized **3** to produce highly oxidative catechol-quinone. The quinone oxidized pyrogallol-type catechins and the quinone was reduced to **3**.[27] Therefore, **3** decreased gently. Theasinensins (**5**–**7**), theacitrins (**8**, **9**) (**Figure 3B**), and theaflavins (**10**–**13**) (**Figure 3C**) increased and reached plateaus at 90–120 min when the four monomeric catechins were almost consumed. TRs were quantified by measuring the area of the broad hump on a baseline in a range of t_R 15–50 min (**Figure S4**). The production of the TRs commenced at the beginning of the aeration and continued to increase after the monomers and dimers reached the plateau phase. When the broad hump on the baseline was divided into three blocks, t_R 15–24 min, 25–34 min, and 35–50 min, the area of 15–24 min increased and reached a plateau at 120 min. In contrast, the area of 35–50 min continued to increase after 120 min (**Figure 3D**). The

285 continuous increase in the TRs may reflect the compositional and structural changes of
286 TRs accompanied by the change in UV–vis absorptions. To confirm this assumption, the
287 tea leaf powders aerated for 60, 120, and 180 min were separately extracted, and the
288 polyphenol fractions were partitioned between H_2O and a mixture of EtOAc and hexane
289 (4:1) (**Figure S5**).[35] Further, the aqueous layers containing TRs were compared by
290 reversed-phase and size-exclusion HPLC (**Figure 4**). The HPLC profiles indicated that
291 the TRs with long retention times in reversed-phase HPLC had high molecular weights.
292 This suggested that the molecular size of the TRs increased during the aeration.

293 **Spectroscopic comparison of the TRs of commercial black tea.** Next, the TRs of
294 commercial black tea were examined by ^{13}C NMR spectroscopy. An aqueous acetone
295 extract of black tea was fractionated by Diaion HP20 chromatography, and the TRs
296 detected at 15–35 min ($TR_{15–35\ min}$) and 25–50 min ($TR_{25–50\ min}$) on reversed-phase
297 HPLC were separately purified by size-exclusion chromatography using Sephadex
298 LH-20 with a mixture of 7 M urea and acetone (**Figure 5**).[34] The size-exclusion HPLC
299 of the fractions suggested that the number average molecular mass (Mn) of $TR_{25–50\ min}$
300 was larger than that of $TR_{15–30\ min}$ (18779 and 15463, respectively, estimated based on a
301 calibration curve generated from polystyrene standards) (**Figure S6**). The ^{13}C NMR
302 spectra of $TR_{15–30\ min}$ and $TR_{25–50\ min}$ were virtually superimposable (**Figure 5**). However,
303 the relative signal area of A-ring methine (δ_C 93–100) / C-ring C-4 signals (δ_C 23–30)
304 was different: the ratio of $TR_{25–50\ min}$ (1.98) was smaller than that of $TR_{15–30\ min}$ (2.75).
305 Since C-ring C-4 does not react in catechin oxidation,[2] the smaller integration of the
306 A-ring methine carbons of $TR_{25–50\ min}$ suggested that the C-6 or C-8 of A-ring methine
307 was involved in oligomerization.

308 **Areation of fresh tea leaves with phloroglucinol.** To examine the reactivities of

A-rings in real aration, fresh tea leaves were crushed with phloroglucinol (1,3,5-trihydroxybenzene), which is a simple mimic of the catechin A-ring with the same nucleophilicity.[36] After aration with phloroglucinol was carried out, the leaves were heated at 80°C and extracted with aqueous acetone. The separation of the products by a combination of column chromatography using Sephadex LH-20, Diaion HP20, and Chromatorex ODS afforded two monomeric and four dimeric phloroglucinol adducts, and their structures were determined based on NMR spectroscopy data (**Tables S1 and S2**). The HMBCs of the monomeric products (**14** and **15**) (**Figure 6**) revealed that these products were generated by the nucleophilic 1,2-addition of phloroglucinol to the C-4 carbonyl group of the B-ring quinones of **1** and **2**. This was supported by the preparation of **14** by the enzymatic oxidation of a mixture of **1** and phloroglucinol.[2,27] The ^{13}C NMR spectra of **14** showed small signals attributable to minor isomers, and this observation suggested that **14** existed as an equilibrium mixture of stereoisomers, which were interchangeable via a quinone methide intermediate (**Figure 6**). The dimeric products (**16**–**19**) were the phloroglucinol adducts of dehydrotheasinensins C (**16a**), B (**17a** and **18a**), and A (**19a**), respectively (**Figure 6**).[12,37,38] The phloroglucinol-connected B-rings of these dimers showed the same HMBCs, which were related to those observed for **14** and **15** (**Figure 7**). The differences among **16**–**19** were observed in the chemical shifts of C-ring H-3, reflecting the esterification of galloyl groups (**Table S2**). These products showed characteristic hydroxy proton signals at δ_H 11.95–12.12, which were assigned to phloroglucinol C-6-OH based on their HMBCs to phloroglucinol C-1, C-5, and C-6 carbons. The chemical shifts indicated that these hydroxy protons formed hydrogen bonding with B-ring C-3 carbonyl groups. Furthermore, these signals showed ROESY correlations with C'-ring H-2 and H-4, suggesting that the configurations of the B-ring

C-4 of **16**–**19** were *R* configurations (**Figure 7**). This model aration experiment strongly suggested that the B-ring quinones of the pyrogallol-type catechins and the hydrated quinone structures of dehydrotheasinensins reacted with the A-rings of catechin monomers or their oxidation products, such as theaflavins and theasinensins. Notably, the phloroglucinol adducts of catechol-type catechins **3** and **4** were not isolated in this experiment. Probably, the catechol-quinones were mainly consumed for theaflavin production.[14,15,27]

Degradation of dehydrotheasinensins. Dehydrotheasinensins (**16a**–**19a**) are unstable quinone dimers detected in crushed fresh tea leaves, and they are decomposed on heating to afford theasinensins (**5**–**7**).[11,12] Among the dehydrotheasinensins, the reactions of dehydrotheasinensin A (**19a**) were important because it was produced from the most abundant tea catechin. Previous studies demonstrated that the heating of **19a** afforded **7**, its atropisomer theasinensin D (**7a**), and galloyl oolongtheanin (**20**).[12,38] The decomposition was a redox disproportionation to afford **7** and **7a** as reduction products and **20** as one of the oxidation products. Since the aforementioned model aration with phloroglucinol suggested that the dehydrotheasinensins were involved in TR formation, the time course of the decomposition of **19a** at 60°C was examined (**Figure 8A**). Within a few minutes, **19a** decreased to afford **7**, **7a**, and **20**. Thereafter, in contrast to the stable reduction products (**7** and **7a**), the oxidation product (**20**) gradually decreased and a pigment (**21**) and oligomeric products were increased. The NMR measurements of **21** in typical solvents, such as CD_3OD, afforded only uninterpretable broad signals; therefore, the spectra were obtained in DMSO-d_6 containing TFA-*d* at 70°C (**Table S3**). The HMBCs (**Figure 9**) allowed us to propose the structure of **21** with an anthocyanidin-like moiety. This structure was found to be a tautomer of the degradation product of **20**

previously reported by Yanase et al.[39] Interestingly, the galloyl proton signal of **21** was observed at δ_H 5.71, which was an unusually high field compared to typical galloyl esters (δ_H 6.8–7.1). Additionally, the galloyl ester linkage resisted hydrolysis with tannase or 2% H_2SO_4 (2 h at 100°C). Furthermore, the ROESY spectrum showed the correlations of the galloyl protons with the B'-5 and H-3 of the anthocyanidin-like unit. These observations indicated that the galloyl group was stacked on the positively charged anthocyanidin unit and stabilized the chromophore. The production mechanism is proposed as shown in **Scheme S1**, and it should be emphasized the mechanism contains an intramolecular B–A coupling between B-ring C-4 and A' ring C-8". Pigment **21** was not detected in black tea; however, related anthocyanidin-like moieties possibly existed as chromophores in the molecules of the TRs. The increase in the TR-related oligomeric products in the degradation products of **19a** was visualized in **Figure 8B**, and the increase commenced at the initial stage of the degradation. Since **7** and **7a** did not decrease in this experiment, the participation of these reduction products in oligomerization was limited. The oligomeric products produced from **19a** (**TR$_{19a}$**) were purified by size-exclusion chromatography, and the ^{13}C NMR spectrum was compared with that of the TRs obtained from black tea (**Figure S7**). The spectra were almost superimposable, except for the absence of the signals attributable to catechol-type B-ring carbons in the spectrum of **TR$_{19a}$**. Next, the oligomers (**TR$_{19a,3}$**) were prepared from a mixture of **19a** and **3** under the same conditions, and the ^{13}C NMR spectrum of the **TR$_{19a,3}$** showed the signals of the catechol-type B-rings. The heating of **3** alone did not afford oligomeric products; therefore, the results suggested that the A ring of **3** was incorporated into the oligomerization of **19a**. As shown in **Figure 8B**, the increase in **TR$_{19a}$** was markedly observed in the area of long retention times after 30 min. This

observation suggested that the TR-related oligomers generated at the early stage of the reaction retained reactive quinones or the related structures (**Scheme S1**). To confirm this, dehydrotheasinensin C (**16a**), the desgalloyl analog of **19a**, was heated under the same conditions, and the oligomers were treated with 1,2-phenylenediamine, which selectively reacted with *ortho*-quinone or the related 1,2-diketone structures to afford phenazine or quinoxaline derivatives, respectively. The HPLC profile of the phenylenediamine-treated oligomers (**TR$_{16aPhen}$**) showed a longer retention time than that of the non-treated oligomers (**TR$_{16a}$**) (**Figure 10**). The ^{13}C NMR spectrum of the phenylenediamine-treated oligomers showed the signals arising from phenazine or quinoxaline moieties,[11] indicating the presence of quinones or the related diketone structures in the TR-related oligomers produced from dehydrotheasinensins.

Oligomers in crushed tea leaves. The oligomers generated in crushed fresh tea leaves were shown to have quinone or related structures. The crushed fresh tea leaves were treated by four different methods (**Figure 11**): sample A was extracted with 60% EtOH, and sample B was extracted with 60% EtOH containing 1,2-phenylenediamine and TFA. Sample C was heated in a microwave oven and extracted with 60% EtOH, and sample D was prepared by the treatment of extract C with 1,2-phenylenediamine and TFA. The HPLC analysis of the phenylenediamine-treated tea leaves showed peaks attributable to the phenazine derivatives of dehydrotheasinensins (peaks with "p" in **Figure 11**),[11] and the broad hump arising from the oligomers (**TR$_{phen}$**) was largely shifted to long retention times compared with that of A (**Figures 11A–B**). This change indicated that the oligomers in the aerated leaves retained quinone or the related 1,2-dicarbonyl structures. However, after the heating of the leaves, the treatment with phenylenediamine did not show such dramatic change in the chromatogram (**Figures**

405 **11C–D**), implying that the quinone and the related structures were heat-sensitive and
406 decomposed on heating. The aforementioned results of the model aration experiment
407 with phloroglucinol and the reactivity of dehydrotheasinensins[12] suggested that the
408 quinone and the related structures in the oligomers reacted with the A-rings or
409 underwent redox disproportionation on heating.

410 **Conclusions** This study proposed new oligomerization mechanisms involving TR
411 production. The oxidation of pyrogallol-type catechins **1** and **2** generated B-ring
412 quinones, which reacted with the A-rings of catechins or their oxidation products
413 (**Figure 12**). The importance of the reactions of the dehydrotheasinensins (**16a**–**19a**)
414 also became evident. In addition to the direct coupling with the A-rings, the redox
415 disproportionation of the dehydrotheasinensins generated galloyl oolongtheanin (**20**)
416 and its des-galloyl analog with a cyclopenta-1,2-dione tautomeric structure, which could
417 also bind to the A-rings, as evidenced by the production of the pigment (**21**) (**Scheme**
418 **S1**). Additionally, the production of **21** with an anthocyanidin-like chromophore
419 suggested the presence of related chromophores in the TRs. Furthermore, the oligomers
420 produced in the crushed fresh tea leaves were shown to have heat-sensitive quinones or
421 related diketone structures, which were the reactive sites of further couplings.

422 In this study, the TRs were recognized as oligomeric oxidation products detected as a
423 broad hump on the HPLC baseline, and the TRs were purified by size-exclusion column
424 chromatography using Sephadex LH-20 and aqueous acetone containing a high
425 concentration of urea.[34] However, this recognition are not completely the same as that
426 originally defined by Roberts.[3] The polyphenol fractions with relatively low molecular
427 weights obtained by the size-exclusion chromatography showed broad humps on the
428 HPLC results, and this implied that the TRs were not uniform in structures, molecular

sizes, physicochemical properties, and production mechanisms.[5,40-42] The B–A ring couplings shown in this study may be just one of the TR production mechanisms. The size-exclusion mode HPLC with polystyrene standards estimated that the number average molecular mass of $TR_{25–50\ min}$ obtained from commercial black tea was 18779 (**Figures 5** and **S6**). However, the peak top retention time of the TRs (13.57 min) was approximately the same as that of the dimeric ellagitannin with a molecular weight of 1870 (13.55 min, sanguiin H-6, $C_{82}H_{54}O_{52}$) (**Figure S8**).[31,32] Since the physicochemical properties of the TRs were probably more similar to those of ellagitannins than those of polystyrenes, the major components of the TRs may correspond with the tetramers–hexamers of tea catechins. If the estimation is appropriate, the combinations of the B–A ring couplings shown in **Figure 12** and the dimerization mechanisms shown in **Figure 2** sufficiently explain the production of catechin oligomers composed of numerous structures.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI:

Physicochemical data of **14**–**19**; Nuclear magnetic resonance (NMR) spectroscopic data of **14**–**19** and **21** (Tables S1–S3); HPLC profiles of the enzymatic oxidation products of pure tea catechins (Figure S1) and combinations of catechins (Figure S2); HPLC profiles of the model aration using lyophilized fresh tea leaf powder (Figure S3); Quantification of the TRs by HPLC (Figure S4); HPLC profiles of EtOAc–hexane layers and the aqueous layers of the aqueous acetone extracts of

lyophilized fresh tea leaf powder treated with H_2O (Figure S5); A plausible production mechanism of **20** and **21** from **19a** (Scheme S1); Size-exclusion HPLC of the TRs obtained from commercial black tea (Figure S6); Comparison of the ^{13}C NMR spectra of the TRs from black tea, from **19a**, and from a mixture of **19a** and **3** (Figure S7); Calibration curves for the size-exclusion HPLC obtained using polystyrene standards and dimeric and trimeric ellagitannins (Figure S8); 1D and 2D NMR spectra of **14**–**19** and **21** (Figures S9–S50) (PDF)

AUTHOR INFORMATION

Corresponding Author

Takashi Tanaka − Graduate School of Biomedical Sciences, Nagasaki University, 1-14 Bunkyo-machi, Nagasaki 852-8521, Japan; orcid.org/0000-0001-7762-7432; Phone: +81-95-8192432; Email: t-tanaka@nagasaki-u.ac.jp

Author

Keigo Hashiguchi − Graduate School of Biomedical Sciences, Nagasaki University, 1-14 Bunkyo-machi, Nagasaki 852-8521, Japan.

Sena Terahara − Graduate School of Biomedical Sciences, Nagasaki University, 1-14 Bunkyo-machi, Nagasaki 852-8521, Japan.

Kohei Katayama − School of Pharmaceutical Sciences, Nagasaki University, 1-14 Bunkyo-machi, Nagasaki 852-8521, Japan.

Yosuke Matsuo − Graduate School of Biomedical Sciences, Nagasaki University, 1-14 Bunkyo-machi, Nagasaki 852-8521, Japan; orcid.org/0000-0002-6462-9727; Phone: +81-95-8192434; Email: y-matsuo@nagasaki-u.ac.jp

Yoshinori Saito − Graduate School of Biomedical Sciences, Nagasaki University,

1-14 Bunkyo-machi, Nagasaki 852-8521, Japan; orcid.org/0000-0002-3587-9743;
Phone: +81-95-8192433; Email: saiyoshi@nagasaki-u.ac.jp
Complete contact information is available at:

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Notes

The authors declare no competing financial interest.

ABBREVIATIONS

DMSO, dimethyl sulfoxide; DTSA, dehydrotheasinensin A (**19a**); DTSB, dehydrotheasinensin B (**17a** and **18a**); DTSC, dehydrotheasinensin C (**16a**),; ECD, electronic circular dichroism; FABMS, fast atom bombardment mass spectra; HMBC, heteronuclear single quantum correlation, and heteronuclear multiple bond correlation; HPLC, high-performance liquid chromatography; HRFABMS, high-resolution fast atom bombardment mass spectra; NMR, nuclear magnetic resonance; ROESY, rotating frame Overhauser effect spectroscopy; TCA, theacitrin A (**8**); TCC, theacitrin C (**9**); TFA, trifluoroacetic acid; TLC, thin-layer chromatography; $TR_{15–35\ min}$, TRs detected as a broad hump between t_R 15 min and 35 min on HPLC baseline; TR_{16a}, TRs produced from dehydrotheasinensin C (**16a**); $TR_{16aPhen}$, polymeric products obtained by treatment of TR_{16a} with 1,2-phenylenediamine. TR_{19a}, TRs produced from dehydrotheasinensin A (**19a**); $TR_{19a,3}$, TRs produced from a mixture of dehydrotheasinensin A (**19a**) and epicatechin (**3**); $TR_{25–50\ min}$, TRs detected as a broad hump between t_R 15 min and 35 min on HPLC baseline; TRs, thearubigins.

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632

FIGURE LEGEND

Figure 1. Structures of the black tea polyphenols and an HPLC profile of a commercial black tea (Assam, India).

Figure 2. Reactions possibly involved in the production of oligomers from dimers.

Figure 3. Time course of the relative peak area (/caffeine at 235 nm) of the catechins and their oxidation products during model aration using lyophilized fresh tea leaves. Catechins (A), theasinensins and theacitrins (B), theaflavins (C), and thearubigins (TRs) (D).

Figure 4. Reversed-phase (A) and size-exclusion (B) HPLC profiles of the aqueous layer of the tea leaves aerated for 60, 120, and 180 min.

Figure 5. Reversed-phase HPLC of the TR fractions separated from commercial black tea and their ^{13}C nuclear magnetic resonance (NMR) spectra. A: A-ring, B: B-ring, G: galloyl.

Figure 6. Structures of the phloroglucinol adducts and selected HMBCs of **14** and **15**.

Figure 7. Important HMBCs and ROESY correlations of **16**.

Figure 8. HPLC profiles of the degradation of dehydrotheasinensin A (**19a**) in pH 6 buffer at 60°C (A). Expansion of the broad humps on the baseline attributable to oligomeric products (TR_{19a}) (B).

Figure 9. HMBC and ROESY of pigment **21**.

Figure 10. HPLC profiles and ^{13}C nuclear magnetic resonance (NMR) spectra of the TRs produced from **16a** (TR_{16a}) and the TRs treated with 1,2-phenylenediamine ($TR_{16aPhen}$).

Figure 11. HPLC profiles of the 60% EtOH extracts of crushed fresh tea leaves.

A: aerated tea leaves (4 h) are extracted with 60% EtOH. B: aerated leaves are extracted with 60% EtOH containing 1,2-phenylenediamine and TFA. C: aerated leaves are first heated in a microwave oven and then extracted with 60% EtOH. D: to the extract of sample C (1 mL), 1,2-phenylenediamine (2 mg) and TFA (20 μL) are added, and the mixture is heated at 60°C for 30 min. Samples A–D are analyzed by HPLC. Peaks with p in chromatogram B are attributable to the phenazine derivatives of dehydrotheasinensins **16a**–**19a** (reference 11).

Figure 12. Oligomerization mechanisms of the pyrogallol-type catechins by B–A ring couplings.

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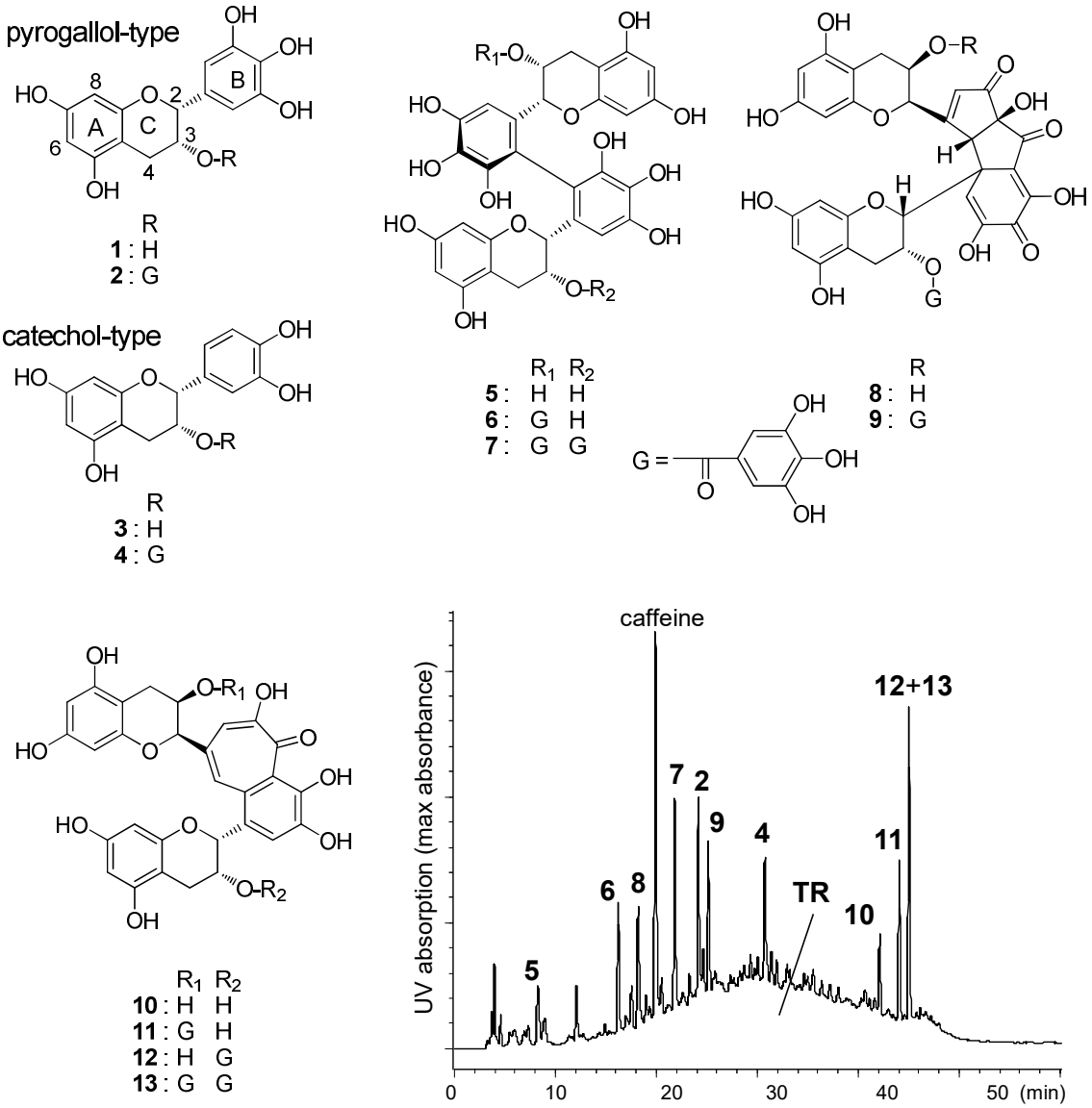

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672 **Figure 1.** Structures of the black tea polyphenols and an HPLC profile of a commercial

673 black tea (Assam, India).

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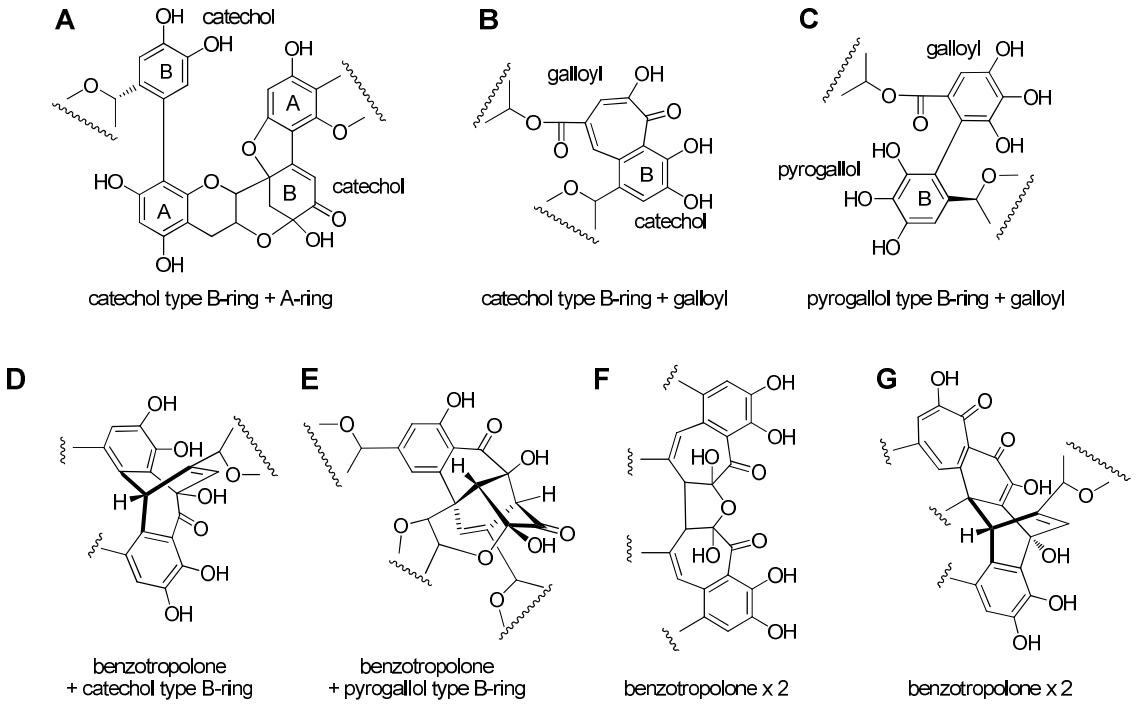

677

678 **Figure 2.** Reactions possibly involved in the production of oligomers from dimers.

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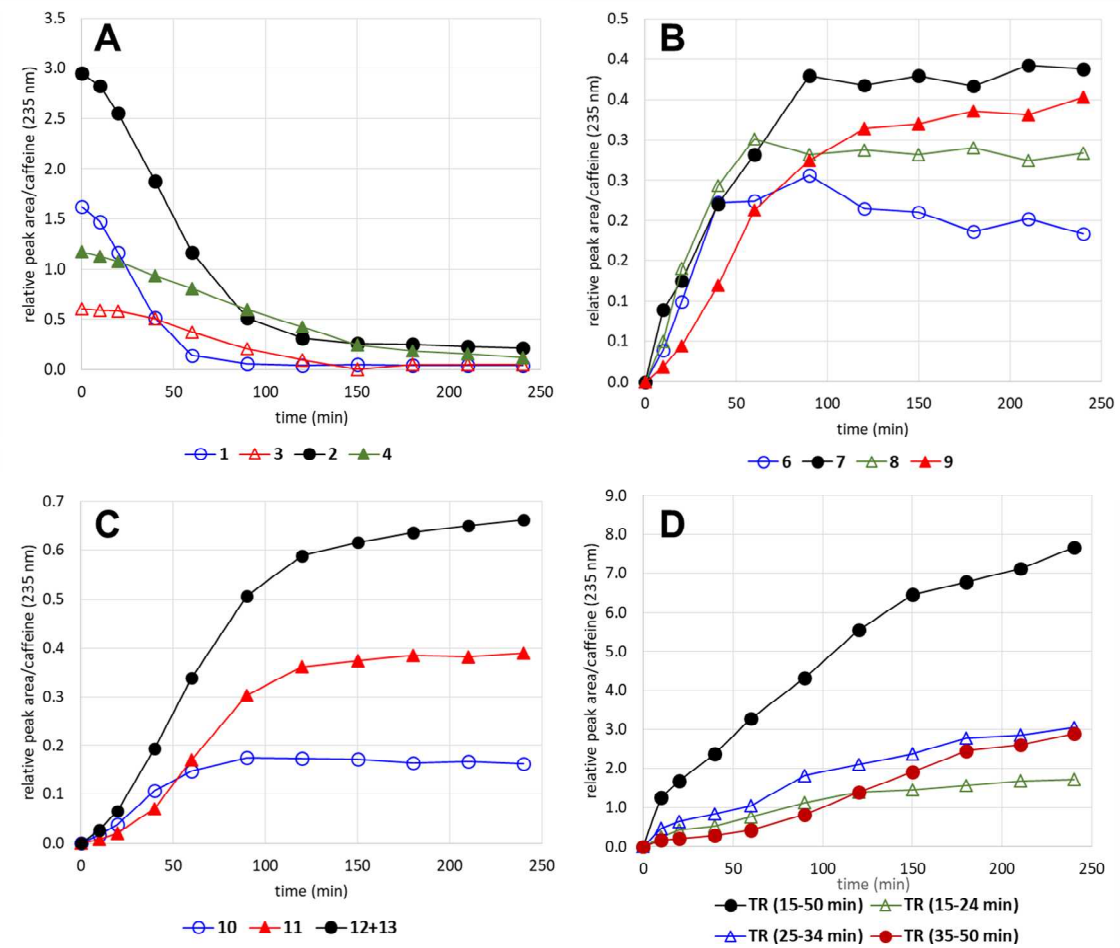

683

684 **Figure 3.** Time course of the relative peak area (/caffeine at 235 nm) of the catechins
685 and their oxidation products during the model high aration using lyophilized fresh tea
686 leaves. Catechins (A), theasinensins and theacitrins (B), theaflavins (C), and
687 thearubigins (TRs) (D).

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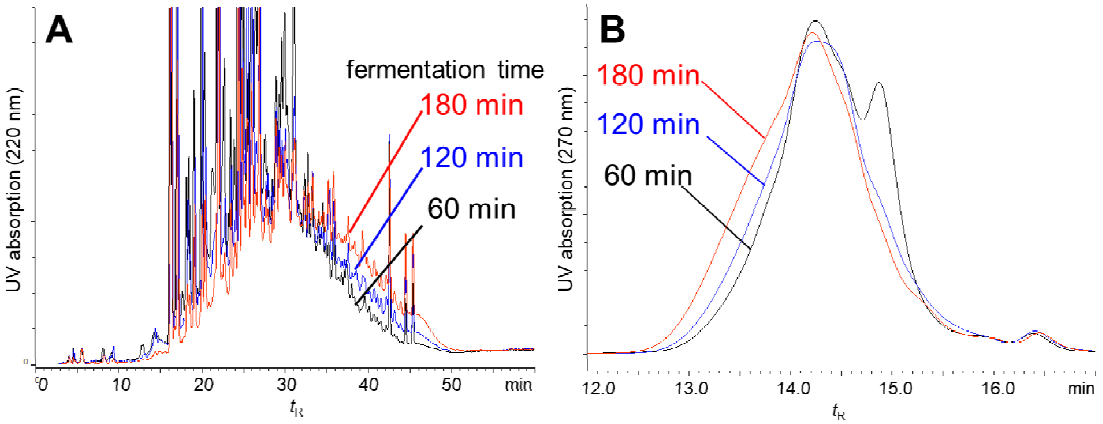

692

693 **Figure 4.** Reversed-phase (A) and size-exclusion (B) HPLC profiles of the aqueous

694 layer of the tea leaves aerated for 60, 120, and 180 min.

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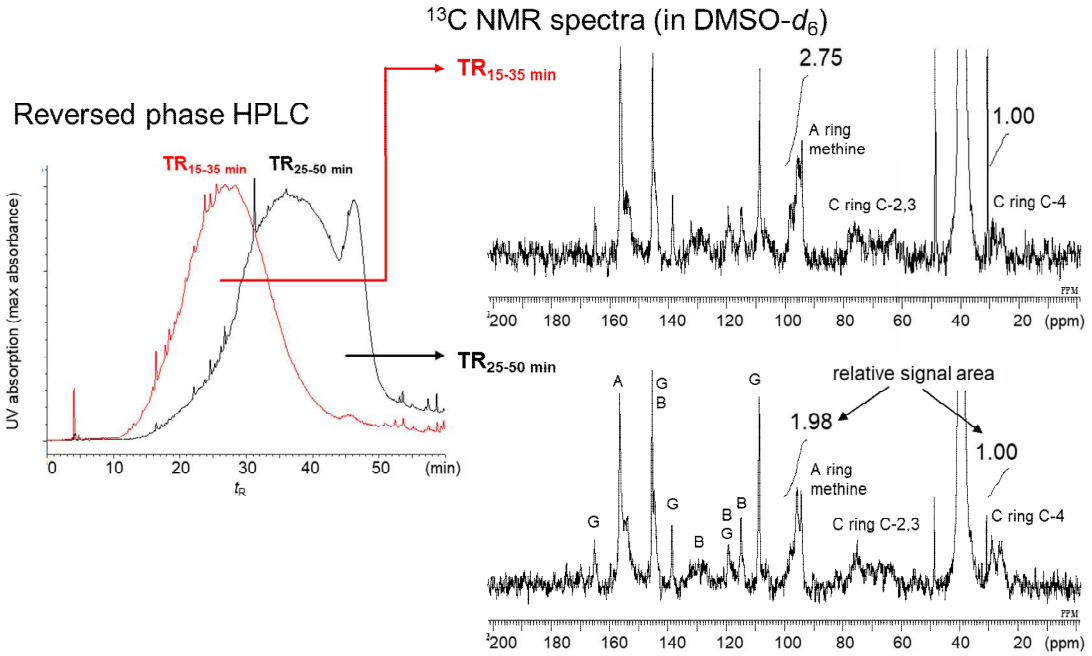

Figure 5. Reversed-phase HPLC of the TR fractions separated from commercial black tea and their ^{13}C nuclear magnetic resonance (NMR) spectra. A: A-ring, B: B-ring, G: galloyl.

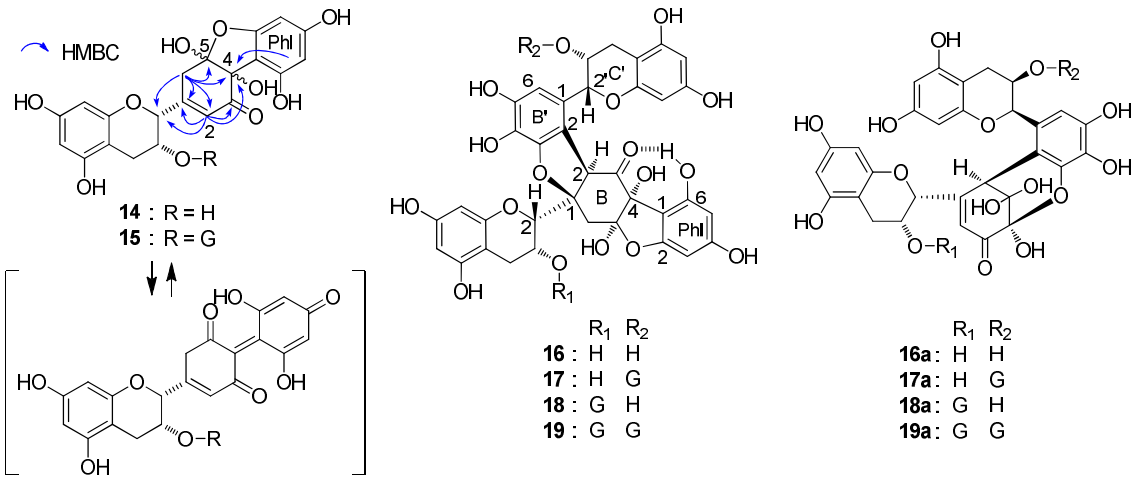

Figure 6. Structures of the phloroglucinol adducts and selected HMBCs of **14** and **15**.

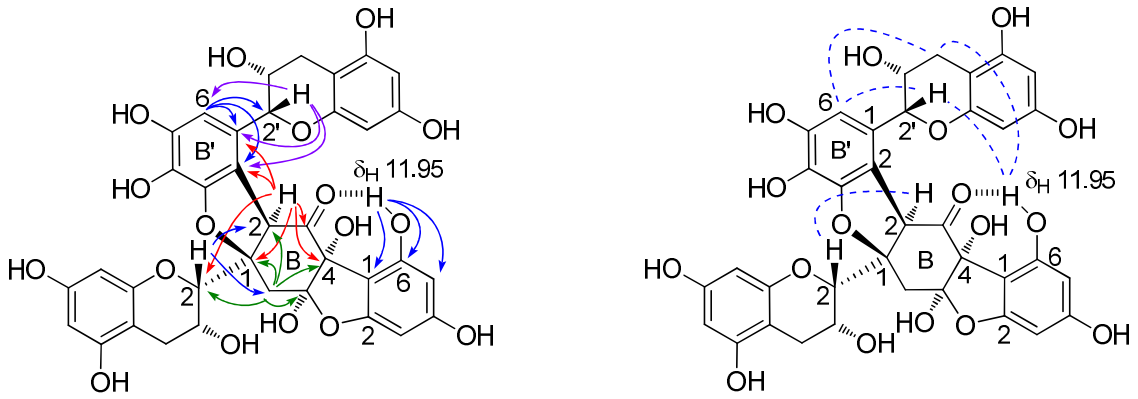

Figure 7. Important HMBCs and ROESY correlations of **16**.

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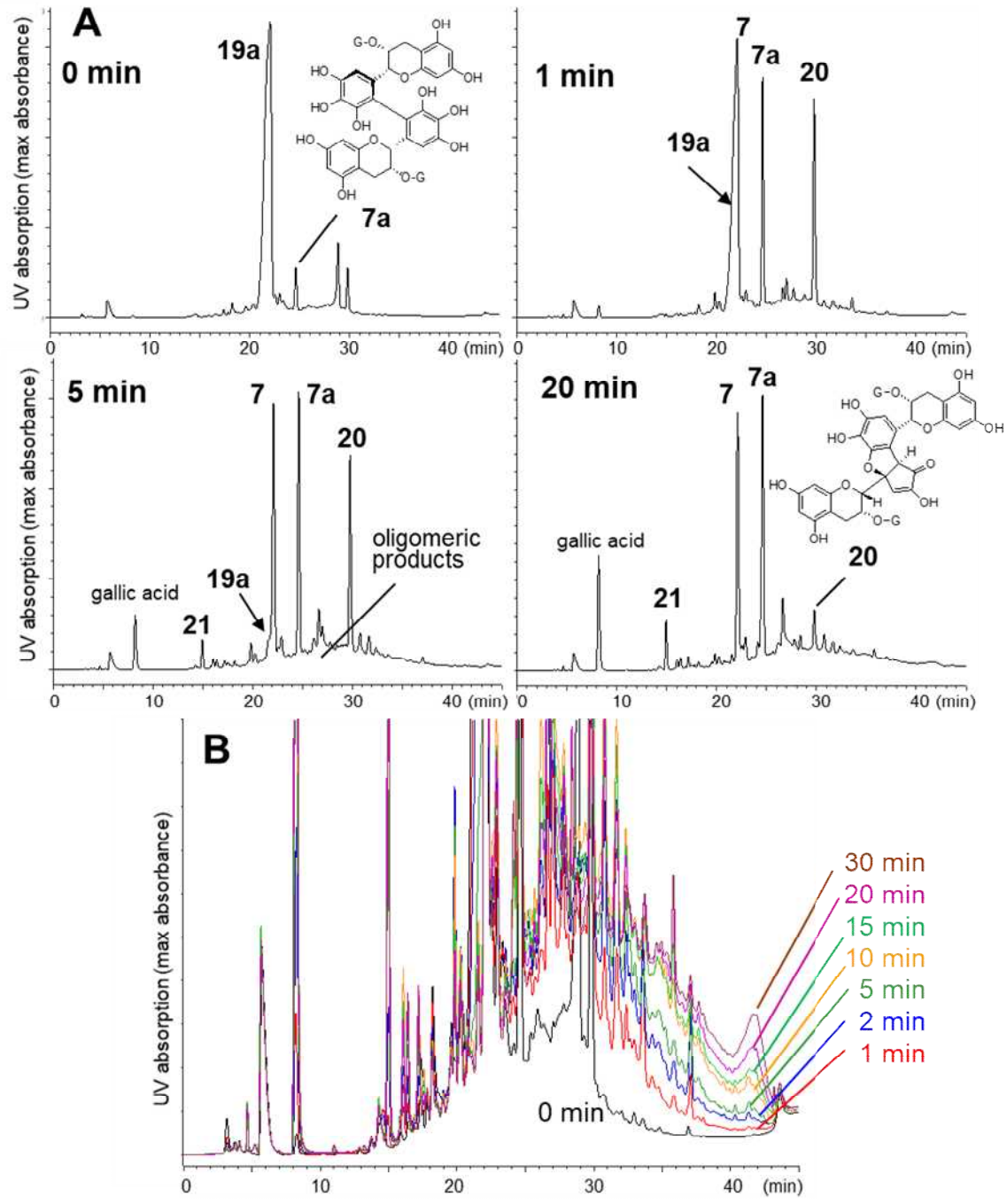
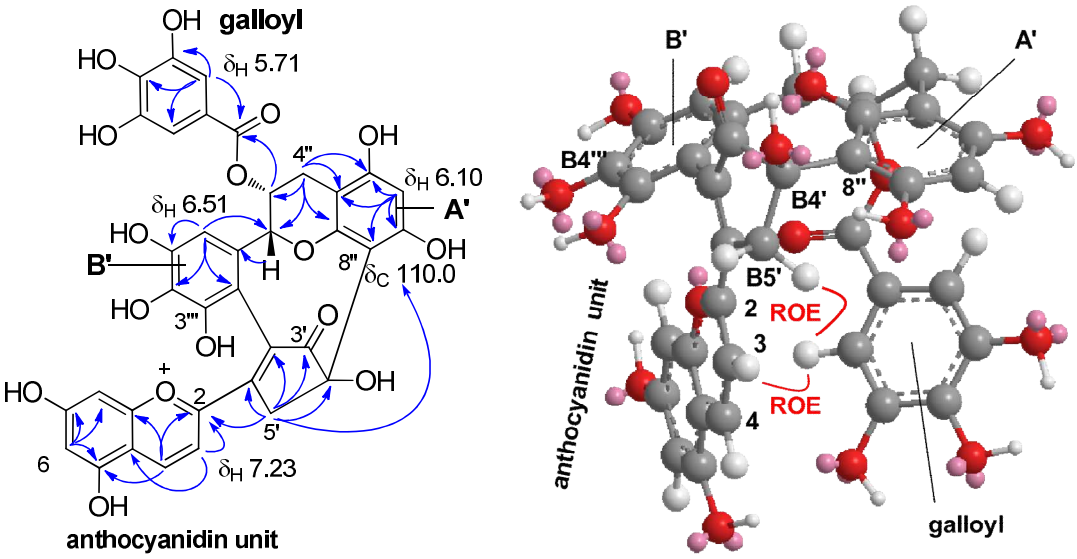


719

720 **Figure 8.** HPLC profiles of the degradation of dehydrotheasinensin A (**19a**) in pH 6
721 buffer at 60°C (A). Expansion of broad humps on the baseline attributable to the
722 oligomeric products (TR_{19a}) (B).



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724 **Figure 9.** HMBC and ROESY of pigment **21**.

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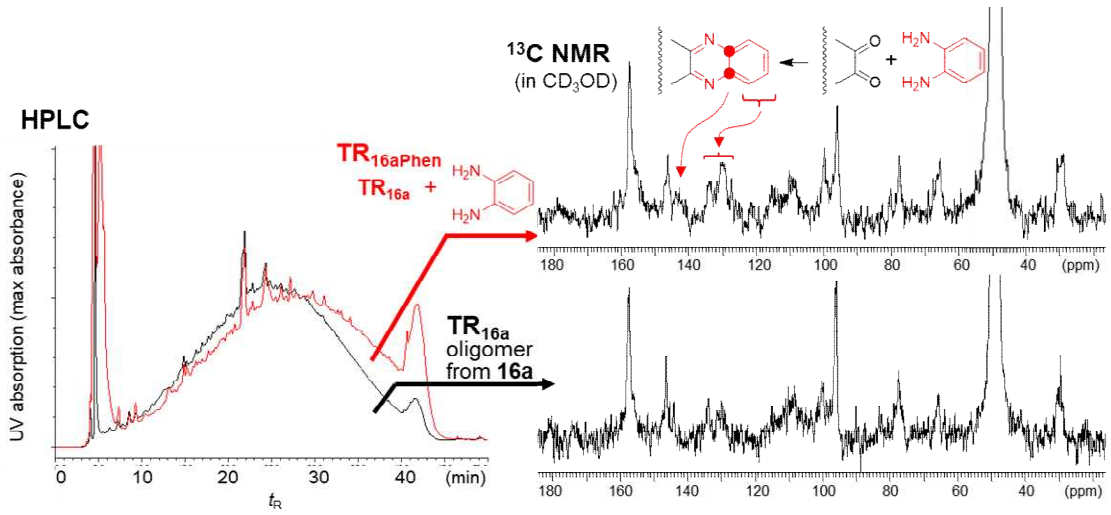

728

729 **Figure 10.** HPLC profiles and ^{13}C nuclear magnetic resonance (NMR) spectra of the

730 TRs produced from **16a** (TR_{16a}) and TRs treated with 1,2-phenylenediamine ($TR_{16aPhen}$).

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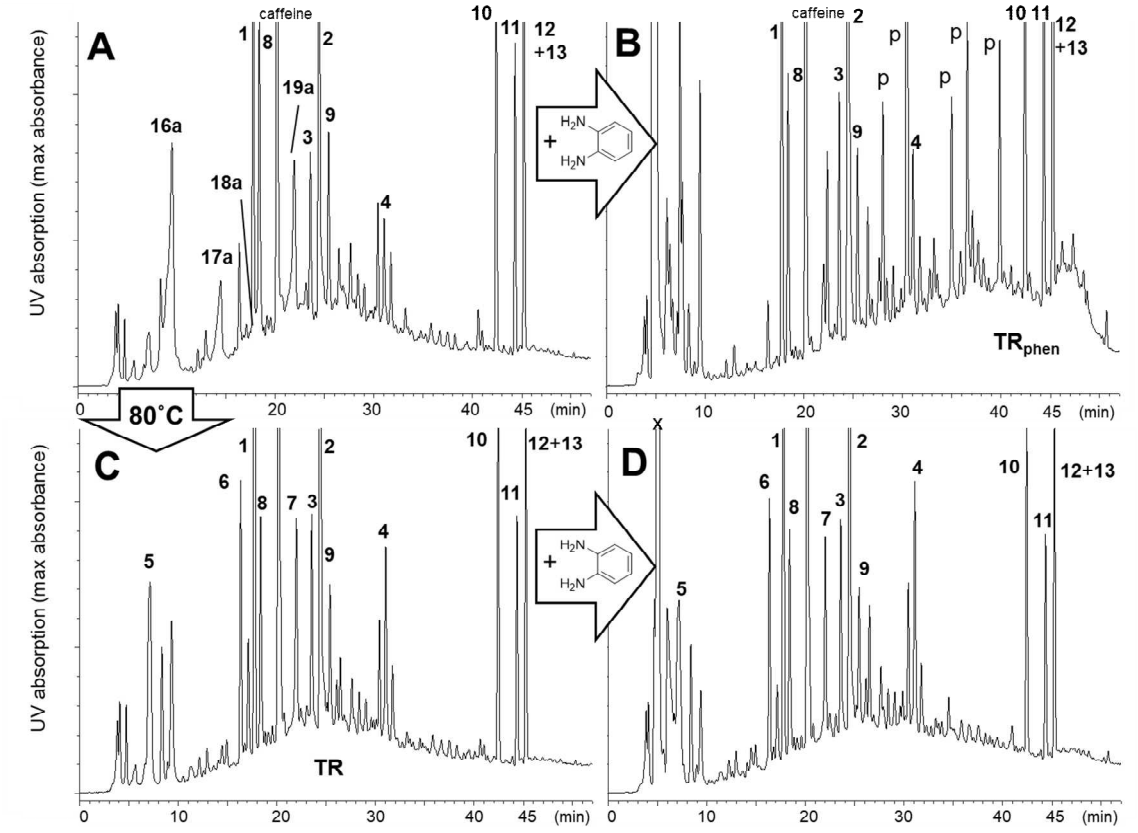

Figure 11. HPLC profiles of the 60% EtOH extracts of crushed fresh tea leaves.

A: aerated tea leaves (4 h) are extracted with 60% EtOH. B: aerated leaves are extracted with 60% EtOH containing 1,2-phenylenediamine and TFA. C: aerated leaves are first heated in a microwave oven and then extracted with 60% EtOH. D: to the extract of sample C (1 mL), 1,2-phenylenediamine (2 mg) and TFA (20 μL) are added, and the mixture is heated at 60°C for 30 min. Samples A–D are analyzed by HPLC. Peaks with p in chromatogram B are attributable to the phenazine derivatives of dehydrotheasinensins **16a**–**19a** (reference 11).

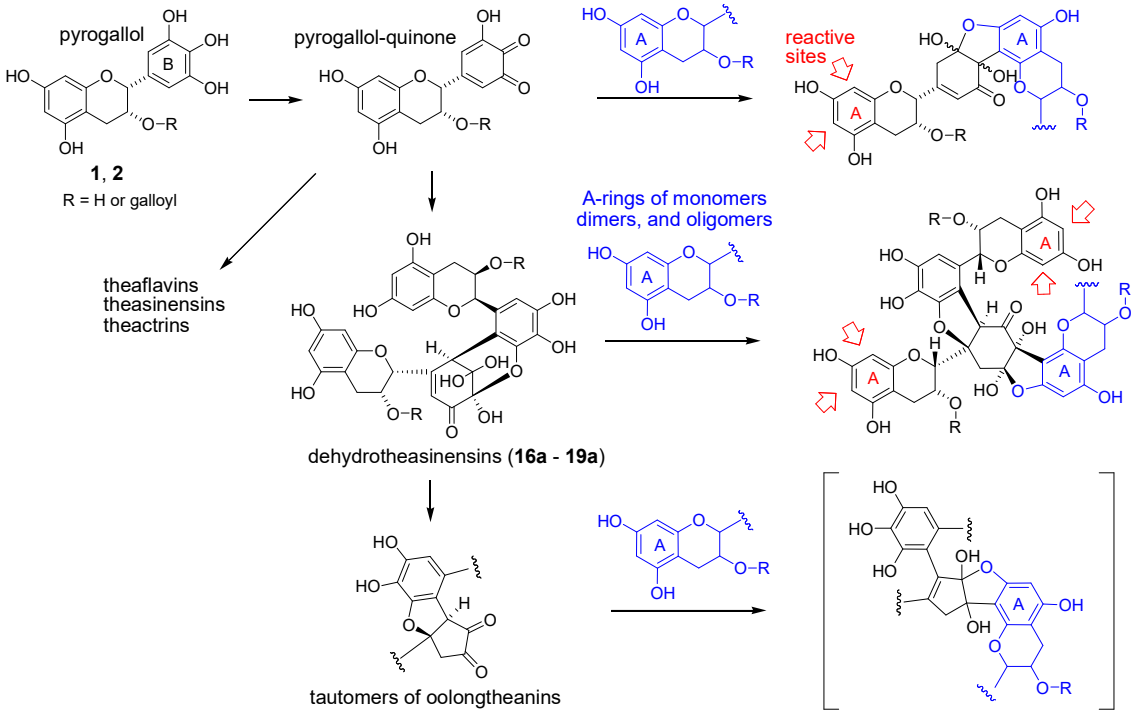

Figure 12. Oligomerization mechanisms of the pyrogallol-type catechins by B–A ring couplings.

754 **TOC graphic**

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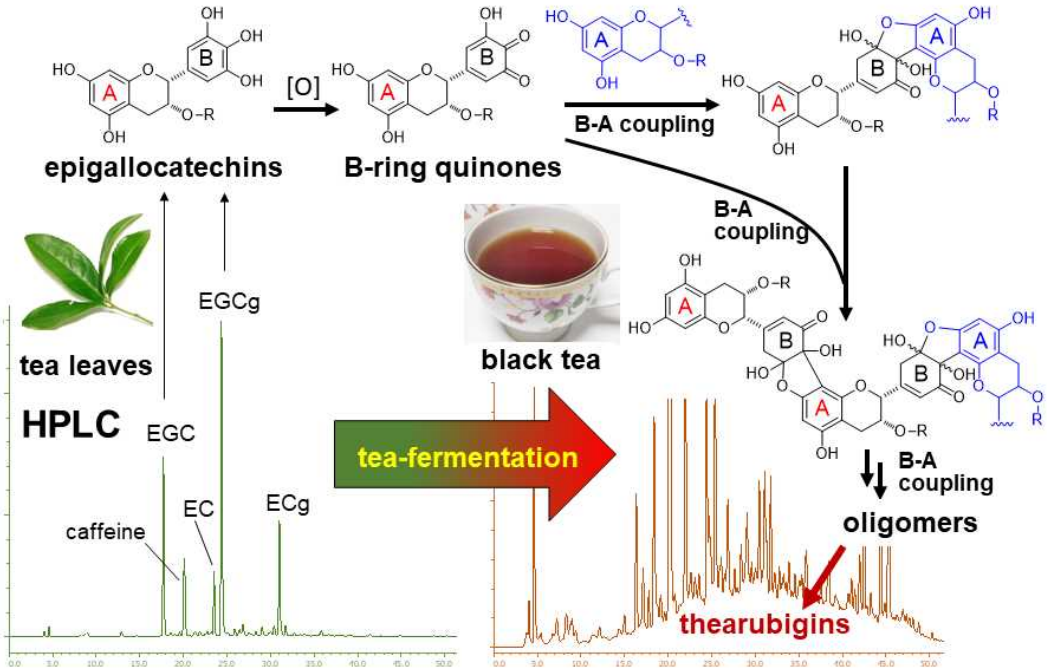

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