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Tannins and Related Compounds. XXXVI.[1] Isolation and Structures of Theaflagallins, New Red Pigments from Black Tea

GEN-ICHIRO NONAKA, FUMIO HASHIMOTO, and ITSUO NISHIOKA*

Faculty of Pharmaceutical Sciences, Kyushu University 62, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812, Japan

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A chemical examination of black tea polyphenols has led to the isolation of three new red pigments, epitheaflagallin 3-*O*-gallate (**1**), epitheaflagallin (**2**) and theaflagallin (**3**), together with the known theaflavins **4**—**7**. The structures of the theaflagallins **1**—**3** have been determined on the basis of spectroscopic and chemical data as unusual benzotropolones formed by oxidative condensation of gallic acid and so-called gallocatechins.

Keywords——black tea; *Camellia sinensis* var. *assamica*; Theaceae; theaflagallin; theaflavin; benzotropolone; red pigment; fermentation; oxidative condensation

The coloring materials of black tea infusions are now well-understood to consist of two types of compounds, theaflavins and thearubigins,[2] both being formed in a fermentation process by endogeneous enzyme oxidation of tea leaf constituents.[3] The chemical nature of the thearubigin pigments still remains elusive, while the theaflavins so far isolated have all been shown to be characteristic disubstituted 1′,2′-dihydroxy-3,4-benzotropolone derivatives formed by oxidative coupling of catechols (catechins) and pyrogallols (gallocatechins and gallic acid).[4]

It is well known that pyrogallol itself readily affords 1′,2′,3′-trihydroxy-3,4-benzotropolone (purpurogallin) when oxidized enzymatically or chemically. Moreover, Takino *et al.* previously prepared flavanotropolonic pigments by coupled oxidation of (−)-epigallocatechin and pyrogallol with tea leaf polyphenol oxidase.[4e] However, until now, no report has appeared on the isolation from black tea of such a condensation product arising from a pair of pyrogallols. We now wish to report on the isolation, characterization and synthesis of a new class of three theaflavin-related pigments, named epitheaflagallin 3-*O*-gallate (**1**), epitheaflagallin (**2**) and theaflagallin (**3**), all of which possess the benzotropolone ring with a 1′,2′,3′-trihydroxy substitution system.

Chromatography of the water-soluble portion of black tea extract (80% aq. acetone) on Sephadex LH-20 (stepwise elution with water containing increasing amounts of methanol) afforded three fractions containing red pigments. The first fraction, which was contaminated with other polyphenolic constituents, was repeatedly chromatographed over Sephadex LH-20, MCI-gel CHP-20P and Bondapak C_{18} to yield compounds **2** and **3**. Similar chromatography of the second and the third fractions gave compounds **4**—**6**, and compounds **1** and **7**, respectively. Compounds **4**—**7** were found to be identical with theaflavin, and its 3-*O*-gallate, 3′-*O*-gallate and 3,3′-di-*O*-gallate, respectively, by comparisons of their physical and spectral data with those described in the literature.[5] Theaflavins 3-*O*-gallate (**5**) and 3′-*O*-gallate (**6**) were formerly obtained as an inseparable mixture, and this is the first time that they have been isolated in pure form.

The molecular formula, $C_{27}H_{20}O_{13}$, of compound **1** was established by elemental analysis and fast atom bombardment mass spectrometry (FAB-MS) (M+H: *m/z* 553). The proton

nuclear magnetic resonance (^{1}H-NMR) spectrum of **1** showed aliphatic proton signals at δ 5.70 (1H, m), 5.22 (1H, s) and 2.86—3.20 (2H, m), which were extremely similar to those of the C-ring protons in (−)-epigallocatechin 3-*O*-gallate (**8**). A two-proton singlet at δ 7.02 (though overlapped with the $C_{6'}$-H signal) and a high-field aromatic singlet at δ 6.14 (2H) were assignable to galloyl and phloroglucinol ring (A-ring of a flavan skeleton) protons, respectively. In the carbon-13 nuclear magnetic resonance (^{13}C-NMR) spectrum of **1**, signals arising from a galloyl group and from the A- and C-rings in a flavan framework closely corresponded to those found in **8**. Apart from these signals, there were eleven carbon signals including a carbonyl (δ 182.4) and sp^2 carbons, and their chemical shifts were consistent with those of purpurogallin (**9**) except for the upfield shift of the C-4 signal in **1**. These ^{13}C-NMR data combined with the observation of three aromatic proton signals at δ 7.02, 7.52 and 7.96, analogous to those found in purpurogallin carboxylic acid (**10**), suggested the presence of a benzotropolone moiety having a substitution pattern similar to that of **10**.

Confirmation of the structure was achieved by the synthesis of compound **1**. Oxidative condensation of **8** and pyrogallol (**11**) with potassium ferricyanide in a weakly alkaline medium[5] yielded, together with **9**, a product shown to be identical with **1**. Similar condensation reaction of **8** and gallic acid (**12**), which are considered to be precursors in tea leaf, also gave **1**, accompanied with **10**.

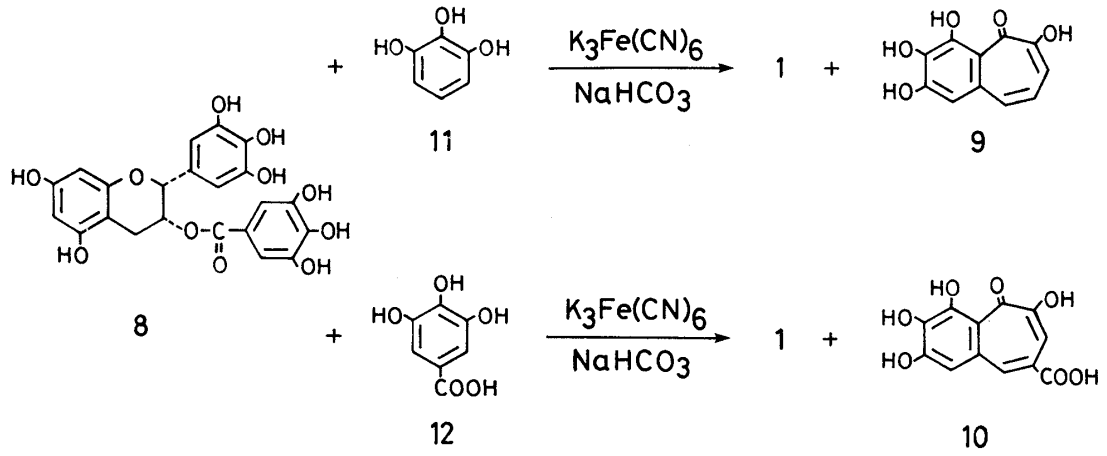
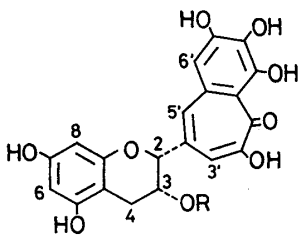
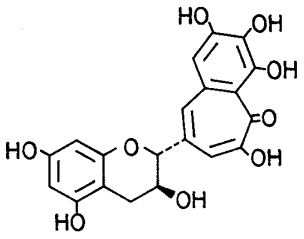


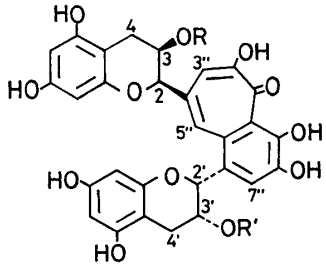
Chart 1



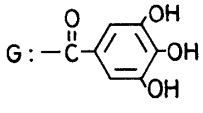
1: R=G
2: R=H



3



4: R=R'=H
5: R=G, R'=H
6: R=H, R'=G
7: R=R'=G



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On the basis of these chemical and spectroscopic data, the structure of compound **1** (epitheaflagallin 3-*O*-gallate) was established as represented by the formula **1**.

The ^{1}H-NMR spectrum of compound **2** was similar to that of **1** except for the absence of the galloyl group and the appearance of the C-3 proton signal in the upper field at δ 4.37 (m), suggesting that **2** is a desgalloyl derivative of **1**. The structure of compound **2** was confirmed by enzymatic hydrolysis of **1** with tannase, which yielded **2** and **12**. Thus, compound **2** was characterized as epitheaflagallin. This compound was previously synthesized by enzyme-catalyzed coupling of (−)-epigallocatechin (**13**) and **11**.[4e]

The ^{13}C-NMR spectrum of compound **3** was almost identical to that of **2**, but differed slightly in the C-2 and C-3 chemical shifts. The appearance of the C-2 and C-3 signals (δ 85.6, 67.7) at lower field than those (δ 81.0, 66.3) of **2** suggested that C-2 and C-3 are in the *trans*-configuration.[6] Indeed, the ^{1}H-NMR spectrum of **3** showed the C-2 and C-3 proton signals at δ 4.56 (d) and 4.06 (m), having large coupling constants of J=9 Hz and $J_{w/2}$=16 Hz, respectively. From these spectral data, compound **3** was considered to be an epimer of **2**, and this was confirmed by similar ferricyanide oxidation of a mixture of (±)-gallocatechin (**14'**) and **11** to give optically inactive **3**. The absolute configurations at C-2 and C-3 remain unclear,

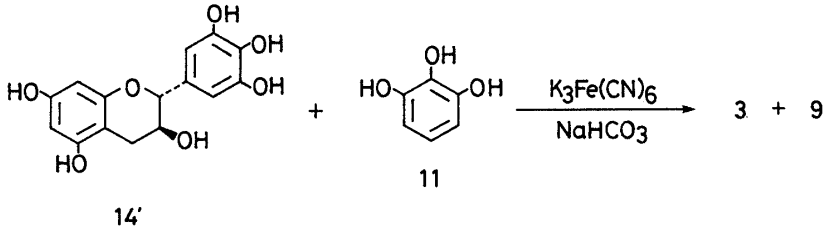

Chart 2

but are supposed to be 2*R* and 3*S* on the ground that (+)-gallocatechin (**14**) is commonly found in nature.

It has been shown that tea leaf pyrogallols, especially the commonly occurring (−)-epigallocatechin (**13**) and gallic acid (**12**), are enzymatically transformed, either by oxidative coupling with catechols into theaflavins,[4] or by oxidative self-condensation into theasinensins[7] and hexahydroxydiphenic acid.[8] The isolation of theaflagallins **1**—**3** shows that another enzymatic oxidation system for pyrogallols exists in tea leaf.

Experimental

Melting points were determined on a Yanagimoto micro-melting point apparatus and are uncorrected. Optical rotations were measured with a JASCO DIP-4 digital polarimeter, ^{1}H- and ^{13}C-NMR spectra were taken with JEOL PS-100 and FX-100 spectrometers, respectively, with tetramethylsilane as an internal standard, and chemical shift values are expressed in δ (ppm). Field desorption (FD)- and FAB-MS were recorded on a JEOL DX-300 spectrometer. Column chromatography was performed with Sephadex LH-20 (Pharmacia Fine Chemicals, 25—100 μ), MCI-gel CHP-20P (Mitsubishi Chemical Industries Ltd., 75—150 μ), Bondapak C_{18}/Porasil B (Waters Associates, 30 μ), and Kieselgel 60 (Merck, 70—230 mesh). Thin-layer chromatography (TLC) was conducted on precoated Kieselgel 60 F_{254} plates (Merck, 0.2 mm thick) with benzene–ethyl formate–formic acid (1 : 5 : 2 or 1 : 7 : 2) and precoated cellulose F_{254} plates (Merck, 0.1 mm thick) with 2% acetic acid, and spots were visualized by spraying 2% ethanolic ferric chloride or by spraying 10% sulfuric acid followed by heating.

Extraction and Isolation——Commercial black tea (a blend of Ceylonese and Indian teas) (2.0 kg) was extracted three times with 80% aq. acetone at room temperature. From the combined extracts, the acetone was removed by evaporation under reduced pressure, and the resulting aqueous solution was shaken with ether to remove chlorophylls, waxes, *etc.* After concentration, the water solubles were applied to a column of Sephadex LH-20, preswollen with H_2O. Elution with H_2O containing increasing proportions of MeOH afforded five fractions; frs. I (97 g), II (190 g), III (110 g), IV (91 g) and V (11 g). Fractions I and II consisted largely of caffeine contaminated with other polyphenolic constituents such as theogallin, (+)-catechin and gallocatechin, and were not examined further. Chromatography of fraction III on Sephadex LH-20 with a solvent system of EtOH–H_2O–acetone[9] yielded a

TABLE I. ^{13}C-NMR Spectral Data for Compounds **1**—**7**, **9** and **10**[a]

	1	**2**	**3**	**4**	**5**	**6**	**7**	**9**	**10**
Benzotropolone moiety									
1	182.4	182.5	182.8	184.9	184.9	184.9	185.1	182.2	182.6
2	154.2	154.0	154.6	154.5	150.5	150.5	150.8	154.6	153.1
3	117.6	117.2	116.1	123.8[b]	124.0	123.0	123.1	116.3	113.9
4	133.6[b]	134.0[b]	134.6[b]	131.5[c]	131.4[b]	130.1[b]	130.2[b]	123.5	136.7
5	133.4	133.4	133.7	126.7[b]	126.2	126.2	126.1	134.3	137.4
5a	134.1[b]	135.6[b]	135.5[b]	128.6	128.2	128.4	128.1	134.7[b]	124.5
6	111.9	111.8	112.0	134.8[c]	133.5[b]	135.4[b]	134.6[b]	110.3	113.3
7	151.9[c]	152.0[c]	152.2	118.7	117.2	118.8	117.3	151.8[c]	151.3[b]
8	135.4	135.3[b]	135.7[b]	146.0	146.2	148.5	145.7[c]	133.1[b]	130.2
9	152.1[c]	152.2[c]	152.2	146.0	146.2	148.5	146.2[c]	151.6[c]	152.3[b]
9a	115.5	115.7	115.7	121.7	121.5	121.6	121.6	114.9	115.0
Flavan C-ring									
2	79.6	81.0	85.6	76.6[d]	79.6	81.1	74.8[d]		
3	69.3	66.3	67.7	65.1[e]	69.4	66.2	70.0[e]		
4	26.6	29.3	29.6	29.5	26.5	29.2	26.4[f]		
2′				81.1[d]	76.5	75.2	80.1[d]		
3′				66.2[e]	65.3	68.2	68.2[e]		
4′				29.5	29.7	26.4	26.8[f]		

a) Spectra were measured in acetone-d_6 + D_2O at 25.05 MHz, except for **9** and **10** (dimethyl sulfoxide-d_6). *b*—*f*) Assignments may be interchanged in each column.

mixture of red pigments **2** and **3**, which were separated by chromatography over Bondapak C_{18} with H_2O in increasing amounts of MeOH to give pure samples (**2**: 13 mg, **3**: 7 mg). Fraction IV was repeatedly chromatographed over Sephadex LH-20 (80% aq. MeOH, EtOH) and MCI-gel CHP-20P (H_2O–MeOH) to yield theaflavins **4** (38 mg), **5** (33 mg) and **6** (82 mg). Separation of fraction V by repeated chromatography over Sephadex LH-20 (80% aq. MeOH, EtOH) and MCI-gel CHP-20P (H_2O–MeOH) gave compounds **1** (92 mg) and **7** (428 mg).

Compound 1 (Epitheaflagallin 3-*O*-Gallate)——A red crystalline powder (H_2O–MeOH), mp 218—220 °C, $[\alpha]_D^{23}$ −383.2° (c=0.4, acetone). *Anal.* Calcd for $C_{27}H_{20}O_{13}\cdot 2H_2O$: C, 55.10; H, 4.11. Found: C, 54.66; H, 4.03. FAB-MS m/z: 553 $(M+H)^+$. ^{1}H-NMR (acetone-d_6 + D_2O): 2.86—3.20 (2H, m, C_4-H), 5.22 (1H, s, C_2-H), 5.70 (1H, m, C_3-H), 6.14 (2H, s, $C_{6,8}$-H), 7.02 (3H, s, galloyl H and $C_{6'}$-H), 7.52 (1H, s, $C_{3'}$-H), 7.96 (1H, s, $C_{5'}$-H). ^{13}C-NMR: Table I.

Synthesis of 1——a) From (−)-Epigallocatechin 3-*O*-Gallate (**8**) and Pyrogallol (**11**): Solutions of **11** (2.8 g) in H_2O (60 ml) and of potassium ferricyanide (10 g) and $NaHCO_3$ (6 g) in H_2O (60 ml) were added stepwise, separately and simultaneously to an ice-cooled solution of **8** (4.4 g) in H_2O (300 ml). After 1 h of stirring, the reaction mixture was acidified with 2 N HCl and extracted three times with ethyl acetate. The ethyl acetate layer was washed with H_2O, dried over Na_2SO_4 and concentrated by evaporation under reduced pressure. The red residue thus obtained was chromatographed over Sephadex LH-20, and elution with EtOH afforded a product (504 mg) identical to compound **1**, together with purpurogallin (**9**) as orange-red needles (H_2O–MeOH), mp 240—243 °C. ^{1}H-NMR (acetone-d_6 + D_2O): 6.79 (1H, dd, J=10, 9 Hz, C_4-H), 7.00 (1H, s, C_6-H), 7.12 (1H, dd, J=9, 2 Hz, C_5-H), 7.38 (1H, d-like, J=10 Hz, C_3-H). ^{13}C-NMR: Table I.

b) From (−)-Epigallocatechin 3-*O*-Gallate (**8**) and Gallic Acid (**12**): Solutions of **12** (1.7 g) in H_2O (60 ml) and of potassium ferricyanide (6.0 g) and $NaHCO_3$ (3 g) in H_2O were added, separately and simultaneously, to an ice-cooled solution of **8** (2.0 g) in H_2O (200 ml) with stirring. After 40 min, the reaction mixture was treated in the same way as described above to yield compound **1** (38 mg) and purpurogallin carboxylic acid (**10**) (13 mg). **10**: Orange-red needles (H_2O–MeOH), mp 276—278 °C. ^{1}H-NMR (acetone-d_6 + D_2O): 7.20 (1H, s, C_6-H), 7.72 (1H, d, J=1 Hz, C_3-H), 8.29 (1H, d, J=1 Hz, C_5-H). ^{13}C-NMR: Table I.

Enzymatic Hydrolysis of 1 with Tannase——A solution of **1** (30 mg) in H_2O (4 ml) was shaken for 10 min with tannase (2 mg) at room temperature. The reaction mixture was treated with EtOH, and the precipitates formed were filtered off. The filtrate was concentrated by evaporation under reduced pressure, and the residue was applied to a Sephadex LH-20 column. Elution with EtOH yielded gallic acid (**12**) (8 mg) and a hydrolysate which was shown to be identical with compound **2** (14 mg).

Compound 2 (Epitheaflagallin)——Red needles (H_2O–MeOH), mp 170—171 °C (dec.), $[\alpha]_D^{25}$ −150.1° (c=0.2, acetone). *Anal.* Calcd for $C_{20}H_{16}O_9$: C, 60.00; H, 4.07. Found: C, 59.98; H, 4.37. FAB-MS m/z: 401 $(M+H)^+$. ^{1}H-NMR (acetone-d_6): 2.80—3.15 (2H, m, C_4-H), 4.37 (1H, m, C_3-H), 4.95 (1H, s, C_2-H), 6.01, 6.07 (each 1H, d, J=

2 Hz, $C_{6,8}$-H), 7.01 (1H, s, $C_{6'}$-H), 7.53 (1H, s, $C_{3'}$-H), 7.65 (1H, s, $C_{5'}$-H), 14.98 (1H, chelated OH). ^{13}C-NMR: Table I.

Compound 3 (Theaflagallin)——A red crystalline powder (H_2O–MeOH), mp 203—204 °C, $[\alpha]_D^{18}$ −27.5° (c=0.1, acetone). *Anal.* Calcd for $C_{20}H_{16}O_9 \cdot H_2O$: C, 57.42; H, 4.35. Found: C, 57.64; H, 4.15. FD-MS m/z: 400 (M^+). ^{1}H-NMR (acetone-d_6): 2.56 (1H, dd, J=16, 10 Hz, C_4-H), 3.10 (1H, dd, J=16, 6 Hz, C_4-H), 4.06 (1H, m, C_3-H), 4.56 (1H, d, J=9 Hz, C_2-H), 5.95, 6.08 (each 1H, d, J=2 Hz, $C_{6,8}$-H), 7.03 (1H, s, $C_{6'}$-H), 7.28 (1H, d, J=1 Hz, $C_{3'}$-H), 7.55 (1H, d, J=1 Hz, $C_{5'}$-H), 14.95 (1H, chelated OH). ^{13}C-NMR: Table I.

Synthesis of 3——A solution of potassium ferricyanide (1.0 g) and $NaHCO_3$ (0.6 g) in H_2O (10 ml) was added portionwise to an ice-cooled solution of (±)-gallocatechin (**14'**) (300 mg) and pyrogallol (260 mg) in H_2O (100 ml), and the mixture was stirred for 30 min. The reaction products were separated in the same way as described above to yield compounds **3** (16 mg) and **9** (10 mg).

Compound 4 (Theaflavin)——A red crystalline powder (H_2O–MeOH), mp 243—246 °C, $[\alpha]_D^{25}$ −246.1° (c=0.2, acetone). ^{1}H-NMR (acetone-d_6 + D_2O): 2.70—3.15 (4H, m, $C_{4,4'}$-H), 4.34—4.64 (2H, m, $C_{3,3'}$-H), 5.03 (1H, s, C_2-H), 5.74 (1H, s, $C_{2'}$-H), 5.98—6.10 (4H, m, $C_{6,6',8,8'}$-H), 7.55 (1H, s, $C_{3''}$-H), 7.96 (1H, br s, $C_{5''}$-H), 8.03 (1H, s, $C_{7''}$-H), 14.93 (1H, chelated OH).

Compound 5 (Theaflavin 3-*O*-Gallate)——A red crystalline powder (H_2O–MeOH), mp 230—235 °C (dec.), $[\alpha]_D^{23}$ −362.8° (c=0.4, acetone). ^{1}H-NMR (acetone-d_6 + D_2O): 2.80—3.20 (4H, m, $C_{4,4'}$-H), 4.59 (1H, m, $C_{3'}$-H), 5.35 (1H, s, C_2-H), 5.77 (1H, m, C_3-H), 5.80 (1H, s, $C_{2'}$-H), 6.00—6.20 (4H, m, $C_{6,6',8,8'}$-H), 6.92 (2H, s, galloyl H), 7.63 (1H, s, $C_{3''}$-H), 8.01 (1H, br s, $C_{5''}$-H), 8.05 (1H, s, $C_{7''}$-H), 14.79 (1H, chelated OH). ^{13}C-NMR: Table I.

Compound 6 (Theaflavin 3'-*O*-Gallate)——A red crystalline powder (H_2O–MeOH), mp 236—240 °C (dec.), $[\alpha]_D^{25}$ −370.7° (c=0.7, acetone). ^{1}H-NMR (acetone-d_6 + D_2O): 2.82—3.20 (4H, m, $C_{4,4'}$-H), 4.43 (1H, m, C_3-H), 5.10 (1H, s, C_2-H), 5.79 (1H, m, $C_{3'}$-H), 6.02 (1H, s, $C_{2'}$-H), 6.00—6.18 (4H, m, $C_{6,6',8,8'}$-H), 6.95 (2H, s, galloyl H), 7.58 (1H, s, $C_{3''}$-H), 8.01 (1H, br s, $C_{5''}$-H), 8.07 (1H, s, $C_{7''}$-H), 14.93 (1H, chelated OH). ^{13}C-NMR: Table I.

Compound 7 (Theaflavin 3,3'-Di-*O*-gallate)——A red crystalline powder (H_2O–MeOH), mp 225—228 °C (dec.), $[\alpha]_D^{18}$ −540.9° (c=0.3, acetone). ^{1}H-NMR (acetone-d_6 + D_2O): 2.90—3.25 (4H, m, $C_{4,4'}$-H), 5.47 (1H, s, C_2-H), 5.70—5.85 (2H, m, $C_{3,3'}$-H), 6.08—6.20 (5H, m, $C_{2',6,6',8,8'}$-H), 6.97, 7.02 (each 2H, s, galloyl H), 7.75 (1H, s, $C_{3''}$-H), 7.98 (1H, br s, $C_{5''}$-H), 8.03 (1H, s, $C_{7''}$-H), 14.89 (1H, chelated OH). ^{13}C-NMR: Table I.

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