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Tannins and Related Compounds. LVI.¹⁾ Isolation of Four New Acylated Flavan-3-ols from Oolong Tea. (1)

FUMIO HASHIMOTO, GEN-ICHIRO NONAKA, 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 the aqueous acetone extract of commercial oolong tea has led to the isolation of four new acylated flavan-3-ols **1**—**4**, together with the known compounds **5**—**13**. On the basis of chemical and spectroscopic evidence, compounds **1**—**4** have been characterized as (–)-epiafzelechin 3-*O*-gallate (**1**), (–)-epicatechin 3-*O*-(4-*O*-methyl)-gallate (**2**), (–)-epicatechin 3-*O*-*p*-hydroxybenzoate (**3**) and (–)-epigallocatechin 3-*O*-cinnamate (**4**).

Keywords—oolong tea; *Camellia sinensis* var. *assamica*; Theaceae; acylated flavan-3-ol; tea catechin; tannin

In the course of our systematic chemical examination on polyphenolic constituents in various beverage teas, we previously demonstrated the occurrence of hydrolyzable tannins in green tea,²⁾ and of benzotropolone-type red pigments (theaflagallins) in black tea,³⁾ together with several proanthocyanidin gallates and a new class of dimeric flavan-3-ol gallates (theasinensins A and B) in fresh green tea leaf.⁴⁾ We now wish to report on the isolation and characterization of four new acylated flavan-3-ol derivatives from oolong tea, which is semi-fermented before being dried (the fermentation stage is considered to be intermediate between those in green tea and black tea).

Preliminary examination of several varieties of commercial oolong teas by thin-layer chromatography (TLC) revealed that, among others, the variety (commercial name: Shiraore, 白折) produced in Fukien (福建) province (China) contains relatively large amounts of polyphenols, though the component patterns in these varieties are closely related to each other. We have therefore chosen this commercial sample as a representative for examination of the polyphenolic constituents.

The extraction and isolation procedures are summarized in Chart 1. Among the thirteen phenolic compounds **1**—**13** isolated here, compounds **5**—**13** were found to be identical with (+)-catechin (**5**),⁴⁾ (–)-epicatechin (**6**),⁴⁾ (–)-epigallocatechin (**7**),⁴⁾ (–)-epicatechin 3-*O*-gallate (**8**),⁴⁾ (–)-epigallocatechin 3-*O*-gallate (**9**),⁴⁾ (–)-epicatechin 3-*O*-(3-*O*-methyl)-gallate (**10**),⁴⁾ (–)-epigallocatechin 3-*O*-*p*-coumarate (**11**),⁴⁾ (–)-epigallocatechin 3,3'-di-*O*-gallate (**12**)⁴⁾ and (–)-epigallocatechin 3,4'-di-*O*-gallate (**13**).⁴⁾

Compound **1**, an off-white amorphous powder, $[\alpha]_D -181.7^\circ$ (acetone), $C_{22}H_{18}O_9 \cdot H_2O$, was positive to the ferric chloride and anisaldehyde-sulfuric acid reagents (blue and orange colorations, respectively), suggesting the presence of a pyrogallol moiety and a flavan-3-ol nucleus. The proton nuclear magnetic resonance (¹H-NMR) spectrum (Table I) showed the presence of a flavan skeleton with a 3,5,7,4'-tetrahydroxy substitution pattern. The appearance of a two-proton singlet at δ 7.03 suggested the presence of a galloyl group. These observations were confirmed by enzymatic hydrolysis of **1** with tannase, which gave (–)-epiafzelechin⁵⁾ and gallic acid. The lowfield shift of flavan 3-H in the ¹H-NMR spectrum of **1**, analogous to that found in **8** or **9**, indicated the location of the galloyl group at this position,

Chart 1

thus establishing the structure of **1** as (–)-epiafzelechin 3-*O*-gallate.

Compound **2**, an off-white amorphous powder, $[\alpha]_D -160.1^\circ$ (acetone), gave a light green coloration on TLC with the ferric chloride reagent, suggestive of the occurrence of a catechol ring in the molecule. The molecular formula, $C_{23}H_{20}O_{10}$, confirmed by elemental analysis and fast atom bombardment mass spectrometry (FAB-MS) $[(M+H)^+ m/z: 457]$,

differs from that of **8** in having an extra CH_2 . The $^1\text{H-NMR}$ spectrum (Table I) of **2** was almost identical with that of **8** except for the presence of a methoxy signal at δ 3.83. Ethylation of **2** with diethyl sulfate and potassium carbonate in dry acetone furnished a hexaethyl ether (**2a**), $[\alpha]_D -138.5^\circ$ (CHCl_3), whose electron-impact mass spectrum (EI-MS) showed prominent fragment peaks at m/z 384 and 223, arising from tetraethoxyflaven and 4-*O*-methyl-3,5-di-*O*-ethyl galloyl cations, respectively. On the other hand, enzymatic hydrolysis of **2** with tannase afforded **6** and 4-*O*-methyl gallic acid. The location of the 4-*O*-methyl galloyl group in the epicatechin nucleus was deduced from the lowfield shift of 3-H in the $^1\text{H-NMR}$ spectrum of **2**. Based on these observations, compound **2** was characterized as (–)-epicatechin 3-*O*-(4-*O*-methyl)-gallate.

TABLE I. $^1\text{H-NMR}$ Spectral Data for Compounds **1**–**4**^{a)}

	1	2	3	4
C-Ring				
C ₂ -H	5.19 (s)	5.16 (s)	5.13 (s)	5.03 (s)
C ₃ -H	5.54 (m)	5.59 (m)	5.55 (m)	5.50 (m)
C ₄ -H	2.79–3.24 (m)	2.89–3.24 (m)	2.79–3.22 (m)	2.75–3.17 (m)
A-Ring				
C ₆ -H	6.05 (d, $J=2$ Hz)	6.07 (s)	6.04 (d, $J=2$ Hz)	6.02 (d, $J=2$ Hz)
C ₈ -H	6.09 (d, $J=2$ Hz)	6.07 (s)	6.08 (d, $J=2$ Hz)	6.09 (d, $J=2$ Hz)
B-Ring				
C ₂ -H	7.38 (d, $J=8$ Hz)	7.09 (d, $J=2$ Hz)	7.07 (d, $J=2$ Hz)	6.63 (s)
C ₃ -H	6.78 (d, $J=8$ Hz)	—	—	—
C ₅ -H	6.78 (d, $J=8$ Hz)	6.87 (d, $J=8$ Hz)	6.76 (d, $J=8$ Hz)	—
C ₆ -H	7.38 (d, $J=8$ Hz)	6.93 (dd, $J=8, 2$ Hz)	6.89 (dd, $J=8, 2$ Hz)	6.63 (s)
Galloyl				
C _{2''} ,6''-H	7.03 (s)	—	—	—
4- <i>O</i> -Methylgalloyl				
C _{2''} ,6''-H	—	7.00 (s)	—	—
OCH ₃	—	3.83 (s)	—	—
<i>p</i> -Hydroxybenzoyl				
C _{2''} ,6''-H	—	—	7.76 (d, $J=8$ Hz)	—
C _{3''} ,5''-H	—	—	6.84 (d, $J=8$ Hz)	—
Cinnamoyl				
C _{2''} ,6''-H	—	—	—	7.32–7.80 (m)
–CH=CH–	—	—	—	6.21, 7.52 (each d, $J=16$ Hz)

a) Spectra were measured in acetone- d_6 + D_2O at 100 MHz.

5

- 6: $\text{R}_1=\text{R}_2=\text{R}_3=\text{H}$
 7: $\text{R}_1=\text{OH}, \text{R}_2=\text{R}_3=\text{H}$
 8: $\text{R}_1=\text{R}_3=\text{H}, \text{R}_2=\text{G}$
 9: $\text{R}_1=\text{OH}, \text{R}_2=\text{G}, \text{R}_3=\text{H}$
 10: $\text{R}_1=\text{R}_3=\text{H}, \text{R}_2=3\text{-MeG}$
 11: $\text{R}_1=\text{OH}, \text{R}_2=p\text{-CA}, \text{R}_3=\text{H}$
 12: $\text{R}_1=\text{OG}, \text{R}_2=\text{G}, \text{R}_3=\text{H}$
 13: $\text{R}_1=\text{OH}, \text{R}_2=\text{R}_3=\text{G}$

TABLE II. ^{13}C -NMR Spectral Data for Compounds 1–4^{a)}

	1	2	3	4
C-Ring				
C ₂	78.0	77.8	77.9	77.8
C ₃	69.7	70.1	69.7	69.7
C ₄	26.4	26.4	26.5	26.5
A-Ring				
C _{4a}	98.6	98.6	98.6	98.6
C _{6,8}	95.5	95.5	95.5	95.6
	96.5	96.5	96.4	96.5
C _{5,7,8a}	157.7 (3C)	156.7	156.7	156.5
		157.3	157.3 (2C)	157.3
		157.4		157.5
B-Ring				
C _{1'}	130.2	130.9	130.9	130.3
C _{2'}	128.8	114.2 ^{b)}	114.7 ^{b)}	106.6
C _{3'}	115.6	145.3	145.3 ^{c)}	146.0 ^{b)}
C _{4'}	157.7	145.3	145.4 ^{c)}	133.0
C _{5'}	115.6	115.7 ^{b)}	115.7 ^{b)}	146.1 ^{b)}
C _{6'}	128.8	118.6	118.7	106.6
Acyl group				
–COO–	166.6	166.3	166.4	167.0
C _{1''}	121.2	126.1	121.7	134.9
C _{2'',6''}	109.9	109.8	132.4	128.9
C _{3'',5''}	145.9	151.1	116.0	129.7
C _{4''}	139.1	140.6	162.8	131.3
OCH ₃	—	60.6	—	—
–CH=CH–	—	—	—	118.6
				146.1

a) Spectra were measured in acetone-*d*₆ + D₂O at 25.05 MHz. b–c) Assignments may be interchanged in each column.

Compound **3**, an off-white amorphous powder, $[\alpha]_{\text{D}} -144.4^\circ$ (acetone), $\text{C}_{22}\text{H}_{18}\text{O}_8 \cdot 4/3\text{H}_2\text{O}$, gave the same coloration as **2** with the ferric chloride reagent. The ^1H -NMR spectrum was also correlated closely with that of **8**, but A_2B_2 -type aromatic signals were found at δ 6.84 and 7.76 (each 2H, d, $J=8$ Hz) instead of a two-proton galloyl singlet. The carbon-13 nuclear magnetic resonance (^{13}C -NMR) spectrum (Table II) showed the presence of a carboxyl group and a *para*-hydroxyphenyl ring, in addition to fifteen carbon resonances assignable to an epicatechin moiety. From these observations, **3** was considered to be (–)-epicatechin 3-*O*-*p*-hydroxybenzoate. Further support for the structure was obtained by field-desorption mass spectrum (FD-MS), which showed the $(\text{M}+\text{H})^+$ ion peak at m/z 411, accompanied by prominent peaks at m/z 272 and 121 arising from flaven and *p*-hydroxybenzoyl cations, respectively. Thus, **3** was concluded to be (–)-epicatechin 3-*O*-*p*-hydroxybenzoate.

Compound **4**, an off-white amorphous powder, $[\alpha]_{\text{D}} -144.1^\circ$ (acetone), $\text{C}_{24}\text{H}_{20}\text{O}_8 \cdot 4/3\text{H}_2\text{O}$, showed, with the ferric chloride reagent, a blue coloration characteristic of a pyrogallol derivative. In the ^1H -NMR spectrum (Table I), the observation of five aromatic multiplets at δ 7.32–7.80 and two *trans*-olefinic doublets at δ 6.21 and 7.52 ($J=16$ Hz) suggested the occurrence of a cinnamoyl group in **4**. In addition, the resonances at δ 2.57–3.17 (2H, m), 5.03 (1H, s), 5.50 (1H, m), 6.02, 6.09 (each 1H, d, $J=2$ Hz) and 6.63 (2H, s) suggested the presence of an epigallocatechin moiety to which the acyl group is attached at the C-3 position. The ^{13}C -NMR data (Table II) were consistent with these ^1H -

NMR observations, showing the presence of (–)-epigallocatechin and cinnamoyl moieties. Ordinary phenolic methylation of **4** yielded the pentamethyl ether (**4a**), $[\alpha]_D^{25} -146.9^\circ$ (CHCl_3), the EI-MS of which exhibited the molecular ion peak at m/z 506, together with significant peaks at m/z 358 and 131 derived from pentamethoxyflaven and cinnamoyl cations, respectively. Based on these results, the structure of **4** was assigned as (–)-epigallocatechin 3-*O*-cinnamate.

In conclusion, although oolong tea is regarded as a partially fermented product in terms of tea manufacture, significant differences from black tea were found in its phenolic pattern, particularly in the contents and compositions of monomeric flavan-3-ol derivatives. Further chemical examination of other polyphenolic constituents in oolong tea is under way.

Experimental

Details of the instruments and chromatographic conditions used in this study were essentially the same as described in the previous paper,⁶⁾ except for the ratio (5:4:1 or 3:6:1) of the solvent system, benzene–ethyl formate–formic acid, used in TLC of free phenolics.

Extraction and Isolation—Commercial oolong tea (commercial name; Shiraore) (5.0 kg) was extracted three times with 80% aqueous acetone at room temperature. The acetone was removed by evaporation under reduced pressure (*ca.* 40 °C), and the resulting aqueous solution afforded dark green precipitates consisting mainly of chlorophylls, which were removed by filtration. The filtrate was applied to a column of Sephadex LH-20, preswollen with H_2O . Elution with H_2O containing increasing amounts of MeOH and then with H_2O –acetone (1:1) afforded five fractions (frs.), frs. I (345 g), II (160 g), III (440 g), IV (382 g) and V (98 g). Fraction I consisted largely of caffeine contaminated with simple phenolics such as theogallin. Rechromatography of fraction II on columns of Sephadex LH-20 and MCI-gel CHP-20P gave compounds **5** (426 mg), **6** (4.8 g) and **7** (4.4 g). Repeated column chromatography of fraction IV over Sephadex LH-20, MCI-gel CHP-20P, Bondapak C_{18} and Fuji gel with various solvent systems furnished compounds **1** (185 mg), **2** (80 mg), **3** (18 mg), **4** (66 mg), **8** (15.9 g), **9** (35.7 g), **10** (353 mg) and **11** (192 mg). Rechromatography of fraction V on columns of MCI-gel CHP-20P, Sephadex LH-20 and Fuji gel afforded an inseparable mixture of compounds **12** and **13** (90 mg).

(–)-Epiafzelechin 3-*O*-Gallate (1**)**—An off-white amorphous powder, $[\alpha]_D^{21} -181.7^\circ$ ($c=0.9$, acetone). *Anal.* Calcd for $\text{C}_{22}\text{H}_{18}\text{O}_9 \cdot \text{H}_2\text{O}$: C, 59.46; H, 4.54. Found: C, 59.96; H, 4.27. FD-MS m/z : 427 $[(\text{M}+\text{H})^+]$, 4%. $^1\text{H-NMR}$: Table I. $^{13}\text{C-NMR}$: Table II.

Enzymatic Hydrolysis of **1 with Tannase**—A solution of **1** (20 mg) in H_2O (4 ml) was shaken for 10 min with tannase (2 mg) at room temperature. The reaction mixture was concentrated by evaporation under reduced pressure and treated with EtOH, and the precipitate formed was filtered off. The filtrate was concentrated, and the residue was applied to a Sephadex LH-20 column. Elution with EtOH yielded gallic acid (4 mg) and a hydrolysate, which was shown to be identical with (–)-epiafzelechin (7 mg).

(–)-Epicatechin 3-*O*-(4-*O*-Methyl)-gallate (2**)**—An off-white amorphous powder, $[\alpha]_D^{22} -160.1^\circ$ ($c=1.1$, acetone). *Anal.* Calcd for $\text{C}_{23}\text{H}_{20}\text{O}_{10} \cdot 2/3\text{H}_2\text{O}$: C, 58.97; H, 4.59. Found: C, 58.71; H, 4.73. FAB-MS m/z : 457 $[(\text{M}+\text{H})^+]$, 0.7%, 184, 167. $^1\text{H-NMR}$: Table I. $^{13}\text{C-NMR}$: Table II.

Enzymatic Hydrolysis of **2 with Tannase**—A solution of **2** (42 mg) in H_2O (5 ml) was shaken for 10 min with tannase (2 mg) at room temperature. The reaction mixture was worked up as described above to give 4-*O*-methyl gallic acid (5 mg) and **6** (12 mg).

Ethylation of **2**—A mixture of **2** (27 mg), diethyl sulfate (0.4 ml) and anhydrous potassium carbonate (0.5 g) in dry acetone (5 ml) was refluxed for 2 h with stirring. After removal of inorganic salts by filtration, the filtrate was concentrated to a syrup, which was chromatographed over silica gel. Elution with benzene–acetone (50:1, v/v) gave crude **2a**, which was purified by chromatography over a silica gel column with benzene–ethyl acetate (40:1, v/v) to yield the ethyl ether (**2a**) (31 mg) as an off-white amorphous powder, $[\alpha]_D^{18} -138.5^\circ$ ($c=0.6$, CHCl_3). *Anal.* Calcd for $\text{C}_{35}\text{H}_{44}\text{O}_{10} \cdot 3\text{H}_2\text{O}$: C, 61.93; H, 7.43. Found: C, 62.25; H, 7.49. EI-MS m/z : 624 (M^+ , 0.2%), 384, 223. $^1\text{H-NMR}$ (CDCl_3): 1.23–1.51 (18H, m, $6 \times \text{OCH}_2\text{CH}_3$), 3.03 (2H, d, $J=4\text{ Hz}$, $\text{C}_4\text{-H}$), 3.84 (3H, s, OCH_3), 3.84–4.20 (12H, m, $6 \times \text{OCH}_2\text{CH}_3$), 5.09 (1H, s, $\text{C}_2\text{-H}$), 5.59 (1H, m, $\text{C}_3\text{-H}$), 6.08, 6.21 (each 1H, d, $J=2\text{ Hz}$, $\text{C}_{6,8}\text{-H}$), 6.79 (1H, d, $J=8\text{ Hz}$, $\text{C}_5\text{-H}$), 6.96 (1H, dd, $J=8, 2\text{ Hz}$, $\text{C}_6\text{-H}$), 7.01 (1H, brs, $\text{C}_2\text{-H}$), 7.11 (2H, s, $\text{C}_{2',6'}\text{-H}$).

(–)-Epicatechin 3-*O*-*p*-Hydroxybenzoate (3**)**—An off-white amorphous powder, $[\alpha]_D^{21} -144.4^\circ$ ($c=1.0$, acetone). *Anal.* Calcd for $\text{C}_{18}\text{H}_{18}\text{O}_7 \cdot \text{H}_2\text{O}$: C, 59.33; H, 5.53. Found: C, 59.77; H, 5.70. FAB-MS m/z : 347 $[(\text{M}+\text{H})^+]$, 0.7%, 272, 121. $^1\text{H-NMR}$: Table I. $^{13}\text{C-NMR}$: Table II.

(–)-Epigallocatechin 3-*O*-Cinnamate (4**)**—An off-white amorphous powder, $[\alpha]_D^{23} -144.1^\circ$ ($c=1.0$, acetone). *Anal.* Calcd for $\text{C}_{24}\text{H}_{20}\text{O}_8 \cdot 4/3\text{H}_2\text{O}$: C, 62.60; H, 4.96. Found: C, 62.86; H, 4.87. FAB-MS m/z : 437 $[(\text{M}+\text{H})^+]$, 0.7%. $^1\text{H-NMR}$: Table I. $^{13}\text{C-NMR}$: Table II.

Methylation of 4—A mixture of **4** (37 mg), dimethyl sulfate (0.2 ml) and anhydrous potassium carbonate (0.3 g) in dry acetone (5 ml) was refluxed for 1 h with stirring. The reaction mixture was worked up in the same way as described for **2** to give the methyl ether (**4a**) (22 mg) as an off-white amorphous powder, $[\alpha]_D^{18} - 176.9^\circ$ ($c=0.9$, CHCl_3). *Anal.* Calcd for $\text{C}_{29}\text{H}_{30}\text{O}_8 \cdot 1/3\text{H}_2\text{O}$: C, 67.95; H, 6.03. Found: C, 68.16; H, 5.95. EI-MS m/z : 506 (M^+ , 0.3%), 358, 131. $^1\text{H-NMR}$ (CDCl_3): 3.20 (2H, d, $J=4$ Hz, $\text{C}_4\text{-H}$), 3.78—3.86 (15H, m, $5 \times \text{OCH}_3$), 5.05 (1H, s, $\text{C}_2\text{-H}$), 5.63 (1H, m, $\text{C}_3\text{-H}$), 6.14, 6.27 (each 1H, d, $J=2$ Hz, $\text{C}_{6,8}\text{-H}$), 6.37, 7.58 (each 1H, d, $J=16$ Hz, olefinic-H), 6.73 (2H, s, $\text{C}_{2',6'}\text{-H}$), 7.26—7.48 (5H, m, arom-H).

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