Identification of Two Novel Proanthocyanidins in Green Tea†

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The isolation and structural elucidation of epiafzelechingallate-(4β→8)-epicatechingallate (EAG-4β→8-ECG) and epiafzelechingallate-(4β→6)-epicatechingallate (EAG-4β→6-ECG) in green tea samples are described. The combination of various 2D NMR techniques allowed a full structural determination of the underivatized proanthocyanidins even though broadening of the signals did not allow observation of some key correlations that characterize the location of the interflavonoid linkage. The differences in the NMR spectra of the new compounds allowed formulation of criteria for the discrimination between the 4→6 and 4→8 isomers in this type of compound.

Keywords: *Camellia sinensis; tea; proanthocyanidins; HPLC/MS; NMR; epiafzelechingallate-(4β→8)-epicatechingallate; epiafzelechingallate-(4β→6)-epicatechingallate*

INTRODUCTION

Recently tea polyphenols have attracted considerable interest because of their associated health properties, and these have been summarized in a series of reviews (Balentine et al., 1997; Hollman et al., 1997; Wiseman et al., 1997; Blot et al., 1997). It is apparent, however, that the proanthocyanidin fraction is not mentioned in all of these publications, although it seems likely that they should contribute to the health properties of tea. Even though Bu-Abbas et al. (1997) have stated that the contribution of the catechins to the antimutagenic properties of green tea is limited, an estimation of the contribution of the proanthocyanidins is only possible after a method for their quantification in tea has been developed. A prerequisite for such a study is the isolation and structural elucidation of the various components present. In tea a number of proanthocyanidins have been described by Japanese groups [e.g., Nonaka et al. (1983, 1984) and Hashimoto et al. (1989)]. A previous paper deals with the analysis of proanthocyanidins in tea samples by means of HPLC and HPLC/MS methods (Kiehne et al., 1997). However, from these LC/MS experiments no information is obtained regarding the stereochemistry and the position of the interflavonoid bond. In addition, the NMR spectroscopic identification of these compounds is somewhat complicated due to the hindered rotation about the interflavonoid bond, which causes a doubling of the NMR resonances (two rotamers) or a broadening of the signals (Thompson et al., 1972). Therefore, in earlier work derivatization was considered to be necessary for the identification of the proanthocyanidins as this allowed NMR experiments to be conducted at elevated temperatures to overcome the rotation barriers (Kolodziej, 1986). Balas and Vercauteren (1994) used 2D NMR techniques at ambient temperatures for the assignment of the peracetates.

More recently, the structural elucidation of underivatized proanthocyanidins in acetone-d_6 after the addition of $Cd(NO_3)_2$ has been published (De Bruyne et al., 1996). In this paper we describe the structural elucidation of epiafzelechingallate-(4β→8)-epicatechingallate (EAG-4β→8-ECG) and epiafzelechingallate-(4β→6)-epicatechingallate (EAG-4β→6-ECG), as well as of the known epigallocatechin-(4β→8)-epigallocatechingallate (EGC-4β→8-EGCG) and epigallocatechingallate-(4β→8)-epigallocatechingallate (EGCG-4β→8-EGCG) (Figure 1) (Hashimoto et al., 1989; Nonaka et al., 1983) from green tea samples.

MATERIALS AND METHODS

General Procedures. For isolation a commercially available China green tea (Lung Ching from Zhejiang) has been used. The obtained compounds were identified on the basis of their spectral data (MS, NMR, CD) and the results of thiolytic degradation.

Extraction and Isolation Procedures. The method (Kiehne et al., 1997) proposed for the isolation and separation of proanthocyanidins in tea has been slightly modified. The green tea was extracted using aqueous acetone (75%, v/v), and the proanthocyanidins were separated from the flavanols by polyamide column chromatography. After elution with aqueous acetone and freeze-drying, a proanthocyanidin-rich residue was obtained.

For isolation of **1** and **2** 40 g of tea was used. From the proanthocyanidin-rich residue compounds **1** (8 mg) and **2** (13 mg) were isolated using semipreparative HPLC.

For the isolation of **3** and **4** the freeze-dried residue (0.5 g) was chromatographed with MeOH on a Sephadex LH-20 column (3 × 35 cm; conditioned with MeOH/EtOAc 1:1). Four fractions were collected (280, 100, 150, and 130 mL). **3** was isolated from fraction II and **4** from fraction IV using semipreparative HPLC. Two grams of the freeze-dried extract was separated using this gel chromatographic step, yielding 9 mg of **3** and 6 mg of **4**. After the spectroscopic data were recorded, a thiolytic degradation of every compound was carried out.

Semipreparative HPLC. HPLC was conducted on a Beckman gradient system (high pressure): column, Hypersil,

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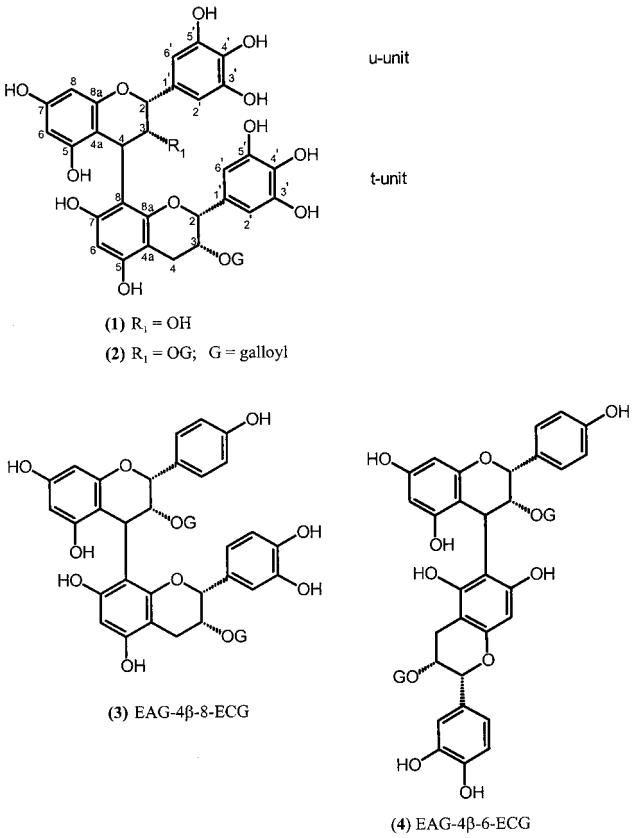

Figure 1. Structures of EGC-4β→8-EGCG (**1**), EGCG-4β→8-EGCG (**2**), EAG-4β→8-ECG (**3**), and EAG-4β→6-ECG (**4**).

5 μm (16 × 250 mm); eluents, 2% acetic acid (aq) and acetonitrile; UV detection at 280 nm.

Acid-Catalyzed Thiolytic Degradation. A mixture of the isolated compound (4 mg), toluene-α-thiol (0.2 mL), and HOAc (0.1 mL) in ethanol (5 mL) was refluxed for 6 h under N_2. The reaction mixture was concentrated under reduced pressure and injected into the HPLC system (Nucleosil 5 μm column, 250 × 4.6 mm; eluent, acetonitrile/2% acetic acid). The configuration of the flavanol formed by the lower unit was verified by on-line coupled polarimetric detection (Chiralyzer polarimetric detector, IBZ Messtechnik, Germany).

*Epigallocatechin-(4β→8)-epigallocatechingallate (**1**):* freeze-dried white powder; thermospray LC/MS (buffer ionization), $[M + H]^+$ *m/z* 763, $[u\text{-unit} - H]^+$ *m/z* 305, $[t\text{-unit} + H]^+$ *m/z* 459; CD (*c* 0.0007 M in MeOH) $[\theta]_{241}$ +4517, $[\theta]_{275}$ −15021; ^{1}H NMR [400 MHz, Me_2CO-d_6, after addition of a trace of Cd-$(NO_3)_2$] δ 8.30−7.50 (broad aromatic OH signals), 8.20 (1H, *s*, OH-5t), 7.12 (broad aromatic OH signal), 7.09 (2H, *s*, galloyl H-2′, H-6′), 6.70 (2H, *br s*, H-2′t, H-6′t), 6.50 (2H, *s*, H-2′u, H-6′u), 6.05, 5.96 (each 1H, *s*, H-6u, H-8u), 6.02 (1H, *s*, H-6t), 5.57 (1H, *s*, H-3t), 5.17 (1H, *br s*, H-2t), 5.14 (1H, *s*, H-2u), 4.85 (1H, *s*, H-4u), 4.02 (1H, *s*, H-3u), 3.47 (1H, *s*, OH-3u), 3.10, 2.97 (each 1H, *dd*, *J* = 17.3, 4.5 Hz, *J* = 17.3, 2.6 Hz, H-4t); ^{13}C NMR (100 MHz, Me_2CO-d_6) δ 166.2 (galloyl C-7′), 155−158 (C-8au, 7u, 5u, 7t), 155.5 (C-5t), 154.1 (C-8at), 146.1 (C-3′u, 5′u), 145.7 (galloyl C-3′,5′), 138.8 (galloyl C-4′), 132.8 (C-1′u), 131.7 (C-4′u), 121.9 (galloyl C-1′), 110.3 (galloyl C-2′,6′), 106.8 (C-8t, C-2′t,6′t), 106.5 (C-2′u,6′u), 99.84 (C-4au), 99.6 (C-4at), 97.3 (C-6t), 96.4, 95.9 (C-8u, C-6u), 78.0 (C-2t), 77.1 (C-2u), 69.7 (C-3u), 69.0 (C-3t), 36.5 (C-4u), 26.6 (C-4t). The thiolytic degradation of **1** furnished (−)-epigallocatechingallate (formed from the lower unit) and epigallocatechin 4-benzyl thioether.

*Epigallocatechingallate-(4β→8)-epigallocatechingallate (**2**):* freeze-dried white powder; thermospray LC/MS (buffer ionization), $[M + H]^+$ *m/z* 915, $[u\text{-unit} - H]^+$ *m/z* 457, $[t\text{-unit} + H]^+$ *m/z* 459; CD (*c* 0.0002 M in MeOH) $[\theta]_{221}$ −76164, $[\theta]_{241}$ +16455, $[\theta]_{281}$ −31901; ^{1}H NMR (Me_2CO-d_6) δ 7.09 (2H, *s*, galloyl H-2′t, H-6′t), 6.97 (2H, *br s*, galloyl H-2′u, H-6′u), 6.52 (2H, *s*, H-2′t, H-6′t), 6.48 (2H, *s*, H-2′u, H-6′u), 6.10 (1H, *s*, H-6t), 6.01, 5.95 (each 1H, *br s*, H-6u, H-8u), 5.59−5.56 (2H, *m*, H-2u, H-3u), 5.47 (1H, *s*, H-3t), 4.95 (1H, *br s*, H-2t), 4.79 (1H, *s*, H-4u), 3.06, 2.85 (each 1H, *dd*, *J* = 17.2, 4.0 Hz, second system not resolved, H-4t); ^{13}C NMR (Me_2CO-d_6) δ 166.2 (galloyl C-7′),155−158 (C-8au, 7u, 5u, 7t, 5t, 8at), 146.1 (galloyl C-3′,5′), 145.8 (C-3′u,5′u), 145.7 (galloyl C-3′,5′), 138.8 (galloyl C-4′), 132.7 (C-1′u), 130.9 (C-4′u), 121.9 (galloyl C-1′), 110.2 (galloyl C-2′,6′), 107.1 (C-8t), 106.8 (C-2′u,6′u, C-2′t,6′t), 98−102 (C-4au, C-4at), 97.0 (C-6t), 96.3, 95.8 (C-8u,C-6u), 75.0 (C-2u, C-3u), 69.2 (C-3t), 33.2 (C-4u), 26.4 (C-4t). Signals of C-2t and C-4′t could not be observed. The treatment of **2** with toluene-α-thiol gave (−)-epigallocatechingallate and epigallocatechin 4-benzyl thioether.

*Epiafzelechingallate-(4β→8)-epicatechingallate (**3**):* freeze-dried white powder; thermospray LC/MS (buffer ionization), $[M + H]^+$ *m/z* 867, $[u\text{-unit} - H]^+$ *m/z* 425, $[t\text{-unit} + H]^+$ *m/z* 443; CD (*c* 0.0003 M in MeOH) $[\theta]_{213}$ +8089, $[\theta]_{230}$ −5680, $[\theta]_{240}$ +23340.2, $[\theta]_{285}$ −37947; ^{1}H NMR (Me_2CO-d_6) δ 7.27 (2H, *d*, *J* = 8.4 Hz, H-2′u, H-6′u), 7.08 (2H, *s*, galloyl H-2′t, H-6′t), 7.01 (2H, *br s*, galloyl H-2′u, H-6′u), 6.87 (1H, *br s*, H-2′t), 6.76 (1H, *br s*, H-6′t), 6.72 (2H, *d*, *J* = 8.5 Hz, H-3′u, H-5′u), 6.60 (1H, *d*, *J* = 8.2 Hz, H-5′t), 6.14 (1H, *s*, H-6t), 5.93, 5.89 (each 1H, *br s*, H-6u, H-8u), 5.71 (1H, *s*, H-2u), 5.52 (1H, *s*, H-3t), 5.46 (1H, *s*, H-3u), 4.96 (1H, *br s*, H-2t), 4.78 (1H, *s*, H-4u), 3.07, 2.87 (1H, *dd*, *J* = 17.3, 4.7 Hz, second system not resolved, H-4t); ^{13}C NMR (Me_2CO-d_6) δ 166.0 (galloyl C-7′), 155−158 (8au, 7u, 5u, 7t, 5t, 8at), 157.7 (C-4′u), 145.8 (galloyl C-3′,5′), 145.1 (C-3′t), 138.8 (galloyl C-4′), 130.7 (C-1′u, C-1′t), 129.2 (C-2′u,6′u), 121.9 (galloyl C-1′), 119.7 (C-6′t), 115.7 (C-3′u,5′u), 114.6 (C-5′t, C-2′t), 110.1 (galloyl C-2′,6′), 107.2 (C-8t), 99.8 (C-4at, C-4au), 97.2 (C-6t), 96.3, 95.9 (C-8u, C-6u), 78.0 (C-3u), 75.8 (C-2u), 68.6 (C-3t), 33.8 (C-4u), 26.6 (C-4t). Signals of C-2t, C-4′u, and C-4′t could not be observed. The thiolytic degradation of **3** gave (−)-epicatechingallate formed from the lower unit.

*Epiafzelechingallate-(4β→6)-epicatechingallate (**4**):* freeze-dried white powder; thermospray LC/MS (buffer ionization), $[M + H]^+$ *m/z* 867, $[u\text{-unit} - H]^+$ *m/z* 425, $[t\text{-unit} + H]^+$ *m/z* 443; CD (*c* 0.0003 M in MeOH) $[\theta]_{207}$ −75586, $[\theta]_{239}$ +71971, $[\theta]_{281}$ −8234, $[\theta]_{302}$ +1989; ^{1}H NMR (Me_2CO-d_6) δ 7.31 (2H, *d*, *J* = 8.4 Hz, H-2′u, H-6′u), 7.10 (1H, *d*, *J* = 1.2 Hz, H-2′t), 7.06, 7.05 (each 2H, *s*, galloyl H-2′, H-6′), 6.92 (1H, *d*, *J* = 8.4 Hz, H-6′t), 6.78 (2H, *s*, H-3′u, H-5′u), 6.76 (1H, *d*, *J* = 2.3 Hz, H-5′t), 6.14, 6.13 (each 1H, *s*, H-8t, H-6/8u), 6.02 (1H, *d*, *J* = 2.2 Hz, H-6/8u), 5.56 (1H, *s*, H-3t), 5.47 (1H, *s*, H-3u), 5.14 (1H, *s*, H-2t), 5.10 (1H, *br s*, H-2u), 4.65 (1H, *s*, H-4u), 3.09, 2.95 (1H, *dd*, *J* = 16.8, n.b. Hz, second system not resolved, H-4t); ^{13}C NMR (Me_2CO-d_6) δ 166.1 (galloyl C-7′), 155−158 (8au, 7u, 5u, 7t, 5t, 8at), 157.9 (C-4′u), 145.9 (galloyl C-3′,5′), 145.6 (C-3′t), 138.6 (galloyl C-4′), 130 (C-1′u, C-1′t), 128.9 (C-2′u,6′u), 121.4 (galloyl C-1′), 119.1 (C-6′t), 115.8 (C-5′t, C-3′u,5′u), 114.9 (C-2′t), 110.2, 110.0 (galloyl C-2′,6′), 107.7 (C-6t), 100.1 (C-4at, C-4au), 97.3, 96.1, 95.8 (C-8t, C-8u, C-6u), 78.1 (C-2t), 74.2 (C-3u), 68.3 (C-3t), 34.7 (C-4u), 27.2 (C-4t). Signals of C-2u and C-4′t could not be observed. The thiolytic degradation of **4** gave (−)-epicatechingallate formed from the lower unit.

Nuclear Magnetic Resonance (NMR). 1D and 2D NMR spectra were recorded at 300 K on a Bruker ARX 400 NMR spectrometer (^{1}H, 400.13 MHz; ^{13}C, 100.62 MHz) locked to the deuterium resonance of solvent, acetone-d_6. 2D ^{1}H-detected one-bond (HMQC, heteronuclear multiple quantum coherence with decoupling during acquisition) and multiple-bond (HMBC, heteronuclear multiple bond correlation) ^{13}C−^{1}H correlations were recorded using standard Bruker software.

Thermospray HPLC/MS. For conditions, see Kiehne et al. (1997).

Circular Dichroism (CD). CD spectra were recorded in MeOH (20 °C) using a Jasco J-710 polarimeter.

RESULTS AND DISCUSSION

The dimeric constitution of compounds **1** and **2** was deduced from MS analysis (Kiehne et al., 1997). Thi-

olytic degradation of the compounds corroborated the presence of an (−)-epigallocatechingallate moiety in the lower unit. A complete assignment was possible on the basis of the NMR and CD data.

In the case of **1** after the addition of $Cd(NO_3)_2$ to the substrate solution (Kiehlmann and Tracey, 1986), the previously broad hydroxyl group protons appeared as sharp signals. Signal assignments were straightforwardly afforded by COSY and HMQC spectra. The key cross-peaks for determining the 4→8-position of the interflavonoid bond was the observation of correlations through three covalent bonds in the lower flavanol subunit between C-4at and H-5OHt and between C-6t and H-5OHt in the HMBC spectrum that established the free C-6t position. The more direct method involving the correlation between H-2t and C-8at (and H-4u and C-8at) reported in De Bruyne et al. (1996) for proanthocyanidin B3 is not applicable here because the relevant proton is broad and it is also possible that in the 2,3-cis configuration the coupling tends to zero. The NMR data are consistent with those reported by Nonaka et al. (1983), but an identification of the interflavonoid linkage based solely on characteristic correlations in the NMR spectra was not described before for this compound.

In the case of **2**, COSY and HMQC spectra allowed the assignment of the signals in the ^1H NMR spectrum, but no information about the position of the interflavonoid bond could be obtained with the approach used for **1**. When $Cd(NO_3)_2$ was added, the OH signals remained broad and no observable correlation between C-6t and H-5OHt could be detected. However, unambiguous identification was possible after tannase treatment as the hydrolysis products of **1** and **2** were identical according to their NMR spectra and, consequently, both compounds must have identical interflavonoid bonds. The positive Cotton effect at 241 nm in the CD spectrum of **1** and **2** confirmed the 4*R* absolute stereochemistry of the upper unit (Barrett et al., 1979).

The mass spectra of both compounds **3** and **4** exhibited ions at *m*/*z* 867, 425, and 443, indicative of a dimer consisting of (epi-)afzelechingallate as upper unit and (epi-)catechingallate as lower unit. In both dimers the 2,3-cis stereochemistry of the monomer units was evident from the small proton couplings $J_{2,3}$ and $J_{3,4}$ between the aliphatic C-ring protons. Treatment of **3** and **4** with toluene-α-thiol produced (−)-epicatechingallate, establishing the 2*R* stereochemistry in the lower unit.

The aliphatic region of the ^1H NMR spectrum of **3** is similar to that of **2**. In **3** the upper B-ring protons (H-2′u, H-6′u) showed a long-range $^1H-^1H$ correlation in the COSY spectrum to H-2u (δ 5.71), and the adjoining well-defined H-3u and H-4u protons were assigned from the relevant correlations in the same spectrum. The observed upfield shift of C-2u at 76 ppm, assigned via an HMQC spectrum, is consistent with a β-orientation of the lower unit (Cai et al., 1991). The positive Cotton effect at 240 nm in the CD spectrum confirmed the 4*R* absolute configuration at the interflavonoid bond (Barrett et al., 1979). In the lower unit of **3** only the $^1H-^1H$ correlations between the H-4t methylene protons and H-3t were observed. However, the position of H-2t was evident in the spectrum as a broad signal (δ 4.96), integrating for one proton, that was consistent with H-2t (δ 4.95) in **2**.

The strong similarity of both the shifts and signal forms of the C-ring and A-ring protons of **3** with those of **2** is convincing evidence that the location of the interflavonoid bond is the same in both cases. Thus, **3** is the novel compound epiafzelechingallate-(4β→8)-epicatechingallate.

The signal patterns in the NMR spectra of **3** and **4** differ significantly even though both compounds have the same dimeric constitution, C-ring 2,3-cis stereochemistry, and 4*R* configuration of the upper unit. In the $^1H-^1H$ COSY of **4** H-4t (δ 3.09) correlates with H-3t (δ 5.56), which in turn shows a cross-peak with the well-defined singlet of H-2t (δ 5.14). This assignment was further corroborated by the long-range correlation between H-2t and H-2′t of the lower B-ring. In contrast to **3**, no correlations could be detected in the COSY spectrum of **4** between the C-ring protons of the upper system, primarily because of the substantial broadening of H-2u and H-3u. The signals of H-3u and H-4u (δ 5.47 and 4.65, respectively) were unambiguously assigned from their correlations (to C-4u and C-3u at δ 34.7 and 74.2, respectively) in the HMQC spectrum and are comparable to the corresponding ones (δ 5.46 and 4.78) in **3**. Consequently, the broad singlet (δ 5.10), integrating for one proton, can be identified as H-2u. Thus, the only possible structure for compound **4** is epiafzelechingallate-(4β→6)-epicatechingallate.

Close inspection of the ^1H NMR data for the four compounds shows that there are systematic variations in the signal line widths as a consequence of selective broadening caused by the hindered rotation about the interflavonoid bond. Thus, in compounds **1**−**3** the proton H-2t and the B-ring protons (H-2′t and H-6′t) are substantially broadened in contrast to the sharp signals found in **4**. The reverse effect appears for H-2u, which is broad in **4** but sharp in **1**−**3**. A similar contrasting effect is observed for the protons of the galloyl group at C-3u in the upper systems of **2** and **3**, which are broad singlets compared to the sharp singlet found in **4**. Thus, although the direct observation of the appropriate long-range $^{13}C-^1H$ correlations affords the safest and most direct probe for the determination of the type of interflavonoid linkage, there are situations in which these are not detectable. In such cases the observations noted above may serve as additional criteria for differentiation between the various interflavonoid linkages in future structural elucidation of related compounds.

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