62 J. Agric. Food Chem. 2011, 59, 62–69
DOI:10.1021/jf1032334

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

Structure Elucidation of Procyanidin Oligomers by Low-Temperature ^{1}H NMR Spectroscopy

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Procyanidin dimers and trimers, needed as reference compounds for biological studies, have been synthesized from various natural sources using a semisynthetic approach and purified by high-speed countercurrent chromatography (HSCCC). In the past, it has been difficult to elucidate the structure of these compounds, especially the determination of the interflavanoid bond. Here, the structure of two B-type procyanidin dimers, with (+)-catechin ((+)-C) in the upper unit, and eight C-type procyanidin trimers, with (−)-epicatechin ((−)-EC) in the upper unit, have been elucidated using low-temperature ^{1}H NMR spectroscopy, as well as circular dichroism (CD) spectroscopy. This is the first time NOE interactions have been used to characterize the interflavanoid linkage in underivatized procyanidin trimers. Complete analyses of procyanidin C1 (−)-EC-4*β*→8-(−)-EC-4*β*→8-(−)-EC, (−)-EC-4*β*→8-(−)-EC-4*β*→8-(+)-C, (−)-EC-4*β*→6-(−)-EC-4*β*→8-(−)-EC, (−)-EC-4*β*→6-(−)-EC-4*β*→8-(+)-C, (−)-EC-4*β*→8-(−)-EC-4*β*→6-(−)-EC, (−)-EC-4*β*→8-(−)-EC-4*β*→6-(+)-C, (−)-EC-4*β*→8-(+)-C-4α→8-(−)-EC, procyanidin C4 (−)-EC-4*β*→8-(+)-C-4α→8-(+)-C, and procyanidin dimers B6 (+)-C-4α→6-(+)-C and B8 (+)-C-4α→6-(−)-EC are presented.

KEYWORDS: ^{1}H NMR; low temperature; procyanidin dimers of (+)-catechin; procyanidin trimers; phloroglucinolysis; CD

INTRODUCTION

Proanthocyanidins, also known as condensed tannins, are widely distributed secondary metabolites occurring mainly in fruits and are found in various beverages. The polydispersity of proanthocyanidins is due to different flavan-3-ol units and different types of interflavanoid bonds as well as the degree of polymerization. The most widely studied proanthocyanidins are the B-type dimeric procyanidins and the more rare C-type trimers, both of which consist exclusively of (+)-catechin and/or (−)-epicatechin subunits linked through C4→C8 or C4→C6 interflavanoid bonds (**Figure 1**) (*1*, *2*).

The daily intake of proanthocyanidins may vary from several tens to several hundreds of milligrams depending on the diet (*3*). Recently, procyanidins have increasingly attracted attention as they show various biological activities that include antioxidative (*4*), anti-inflammatory (*1*, *3*, *5*), anticancer (*1*, *3*, *5*), antiallergic (*6*), anticaries (*7*), and antihypertensive activities (*8*). For studies concerning bioavailability, bioactivity, and metabolism of procyanidins, pure procyanidin dimers and trimers are required. Recently, we have developed a novel semisynthetic approach for the preparation of 3,4-*trans*-configured procyanidin dimers and smaller amounts of trimers in an efficient and cheap way (*9*−*11*).

Evaluation of the physiological functionalities of procyanidins requires unambiguous structural determination. NMR-based approaches have been used to confirm the structure of procyanidins with and without derivatization and require further analysis, for instance, with circular dichroism (CD) spectroscopy and fast atom bombardment mass spectrometry (FAB-MS) (*9*, *12*−*23*). The structure elucidation of procyanidins is still difficult, in particular, the determination of the interflavanoid linkages. Different NMR techniques have the potential of answering the question of junction positions between flavonoid units that include 2D ^{1}H−^{13}C heteronuclear long-range correlations (HMBC), 2D ^{1}H through-space correlations (NOESY and ROESY), and the use of chemical shift parameters. HMBC experiments may provide a way of identifying linkage positions between procyanidin units (*17*, *19*, *21*), as H4 should show through-bond correlations with C7 and C8a of the adjacent unit in the case of a 4→8 linkage and with C5 and C7 of the adjacent unit in the case of a 4→6 linkage. However, C8, C7, and C5 cannot be unambiguously assigned from other long-range correlations without the recourse to chemical shift arguments. In contrast, ^{1}H through-space interactions can provide conclusive evidence of linkage positions as ROESY spectra show strong correlations between B-ring protons (H2′, H6′) and H4 of the adjacent unit for the 4−8 linkage (*24*) and has been reported recently by us for the elucidation of the dimeric procyanidins B3 ((+)-catechin-4α→8-(+)-catechin), B4 ((+)-catechin-4α→8-(−)-epicatechin), B6 ((+)-catechin-4α→6-(+)-catechin), and B8 ((+)-catechin-4α→6-(−)-epicatechin) (*11*).

CD data facilitate the direct assignment of the absolute configuration at the chiral center C4 and, thus, the interflavanoid linkage. The configuration of the interflavanoid bond at C4 can be assigned as α (4S) due to negative Cotton effects at 220−240 nm

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 Published on Web 12/08/2010 © 2010 American Chemical Society

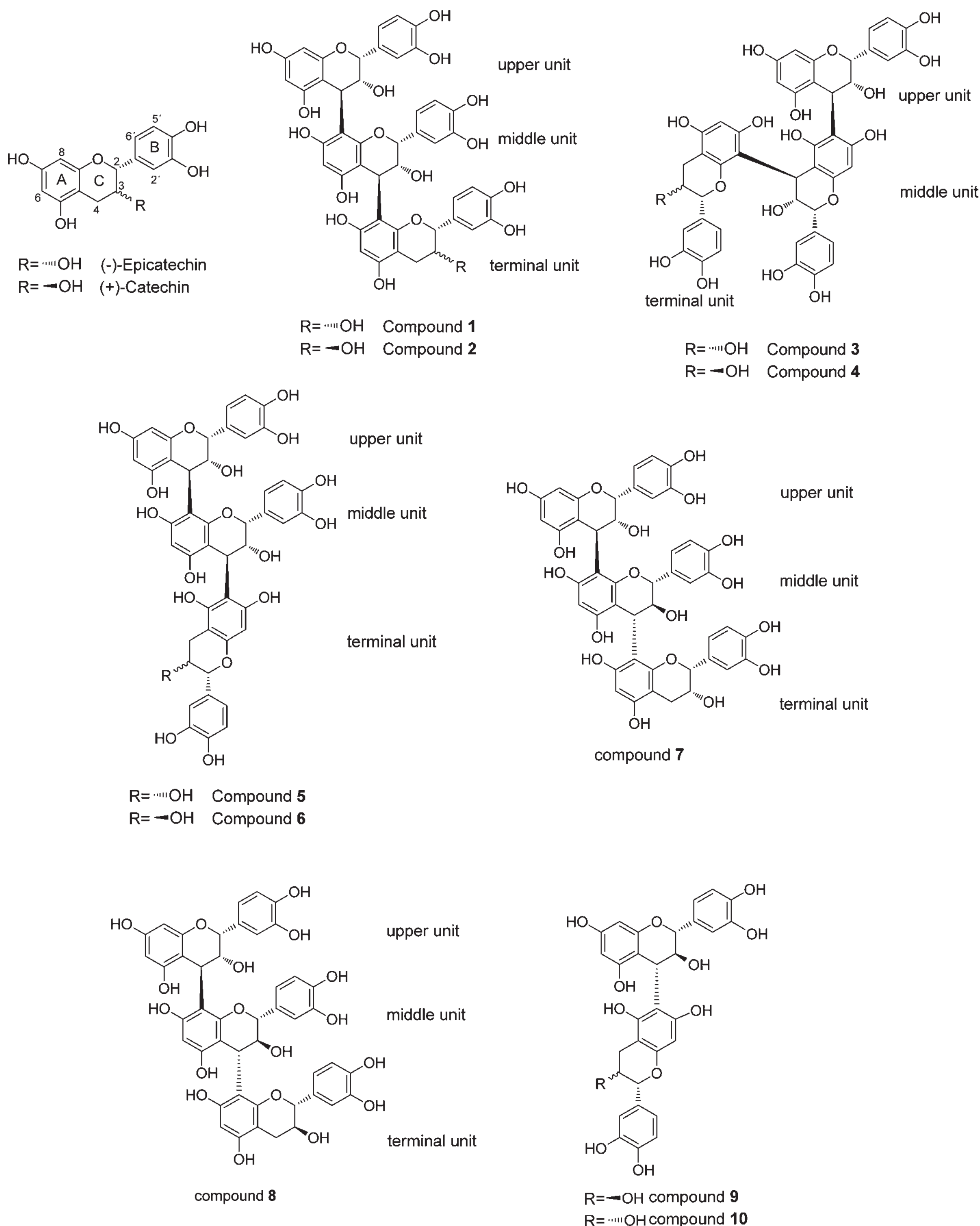

Figure 1. Structures of (−)-epicatechin and (+)-catechin, as well as procyanidin trimers **1**−**8**: procyanidin C1 (−)-EC-4β→8-(−)-EC-4β→8-(−)-EC (**1**), (−)-EC-4β→8-(−)-EC-4β→8-(+)-C (**2**), (−)-EC-4β→6-(−)-EC-4β→8-(−)-EC (**3**), (−)-EC-4β→6-(−)-EC-4β→8-(+)-C (**4**), (−)-EC-4β→8-(−)-EC-4β→6-(−)-EC (**5**), (−)-EC-4β→8-(−)-EC-4β→6-(+)-C (**6**), (−)-EC-4β→8-(+)-C-4α→8-(−)-EC (**7**), and (−)-EC-4β→8-(+)-C-4α→8-(+)-C (**8**) and the 4α→6-linked procyanidin dimers B6 (**9**) and B8 (**10**).

or as β (4R) in the case of positive Cotton effects at 220−240 nm (*16*, *25*−*27*).

Here, we report for the first time a ^{1}H NMR procedure at low temperature (260 or 240 K), without derivatization and ^{13}C NMR measurements, for the structure elucidation of two procyanidin dimers and eight trimers (**Figure 1**). A complete assignment of the ^{1}H NMR data allowed characterization of the nature of the subunits involved ((+)-catechin or (−)-epicatechin) and determination of the position of the interflavanoid bond (4→8 or 4→6). This is the first time NOE interactions have been used to characterize the interflavanoid linkage in underivatized procyanidin trimers. Furthermore, confirmatory evidence of their composition and interflavanoid bond was provided by acid-catalyzed depolymerization in the presence of phloroglucinol. The resulting degradation products, flavan-3-ols and flavan-3-ol adducts, were identified by reversed-phase high-performance liquid chromatography using photodiode

array detection (HPLC-PDA) and electrospray ionization mass spectrometry (ESI/MS).

MATERIALS AND METHODS

Chemicals. The chemicals used and their suppliers were as follows: sodium acetate (anhydrous), p.a. (Merck, Darmstadt, Germany); phloroglucinol, p.a. (Merck); hydrochloric acid, 37% (Riedel-de-Haën, Seelze, Germany); ascorbic acid, pure (Merck); methanol, p.a. (Fisher Scientific, Loughborough, U.K.); methanol for spectroscopy (Uvasol, Merck); acetonitrile, HPLC quality (Fisher Scientific); acetic acid, HPLC quality (Mallinckrodt Baker B.V., Deventer, The Netherlands); water (deionized, Nanopure); methanol-d_4 and acetone-d_6, 99.8% (Deutero GmbH, Kastellaun, Germany).

Preparation of Procyanidin Trimers and Dimers. Compounds **1**−**6** were obtained according to published data by semisynthesis of a polymeric black chokeberry (*Aronia melanocarpa*) fraction (*10*) and compounds **9** and **10** by semisynthesis of a polymeric white willow bark (*Salix alba*) fraction (*11*) as well as enrichment by high-speed countercurrent chromatography (HSCCC). Compounds **7** and **8** were also produced by semisynthesis of a polymeric hazelnut seed coat fraction. Hazelnut seed coat and (+)-catechin or (−)-epicatechin, as nucleophiles, were dissolved in 0.1 N methanolic HCl and kept at 40 °C for 30 min. The reaction mixture was neutralized with 0.5 N sodium hydrogen carbonate solution. After evaporation, the residual aqueous solution was freeze-dried and was applied to countercurrent chromatography separation.

HPLC-PDA Analysis. A HPLC system from Jasco (Gross-Umstadt, Germany), with a PU-2080 plus pump combined with a DG-2080-53 three-line-degasser and LG 2080-02 ternary gradient unit, and MD-2010 plus DAD were used. HPLC conditions were the same as previously described (*10*).

HPLC-ESI/MS/MS Analysis. Chromatographic analyses were performed on an Agilent 1100 HPLC system (Waldbronn, Germany) equipped with a 1200 autosampler and an 1100 HPLC pump. The HPLC was also coupled to the Esquire HPLC-ESI/MS system from Bruker GmbH (Bremen, Germany). HP ChemStation was used for data collection. MS parameters and HPLC conditions were the same as described earlier (*10*).

Preparative HPLC. A HPLC system from Knauer (Berlin, Germany) was used consisting of a Smartline 1000 HPLC pump, a Smartline Manager 5000 solvent organizer and degasser, a Wellchrom K-2600 UV detector, a Rheodyne 7125 injector (200 μL), and ChromGate V3.1.7 software.

The following preparative HPLC columns were used: (a) Hypersil ODS C-18 5 μm, 250 × 16 mm i.d. (Phenomenex, Aschaffenburg, Germany), and (b) Aqua 5 μm C-18, 125 Å, 250 × 21.2 mm i.d. (Phenomenex). Column a was used for the isolation of **1**−**9** and column b for **10**. Water (solvent A) and acetonitrile (solvent B) were used as solvent systems.

Gradient 1 (used for **1**, **3**, **5**, **9**) consisted of initial 10% B increasing in 40 min to 30% B. Gradient 2a (for **2**, **4**) consisted of initial 6% B increasing in 8 min to 13% B and in 32 min to 22% B (the peaks are not pure; hence, purification of these two compounds with gradient 2b, which consisted of initial 8% B increasing in 40 min to 25% B). Gradient 3 (for **6**) began with 10% B, increasing in 10 min to 15% B, 10 min isocratic, and increasing in 30 min to 30% B. Gradient 4 (for **7**) began at 6% B, increasing in 50 min to 25% B. Gradient 5 (for **8**) consisted of initial 1% B increasing in 50 min to 15% B, and gradient 6 (for **10**) began with 10% B increasing in 50 min to 35% B. The flow rate was adjusted to 6.0 mL/min, except for **8** to 5.0 mL/min, and the fractions were monitored by λ = 280 nm. All compounds were isolated as pale white amorphous powders. The purity of each compound was confirmed as > 95% by λ = 280 nm using reversed-phase HPLC-PDA (see above).

Analysis of Isolated Procyanidin Dimers by Phloroglucinolysis. Analysis was carried out according to the method of Kennedy and Jones (*28*). Approximately 0.1 mg of isolated compound was dissolved in 100 μL of reaction solution of 0.1 N HCl in methanol, containing 50 g/L phloroglucinol and 10 g/L ascorbic acid. For incomplete cleavage of the interflavanoid bond (mild degradation), the reaction was carried out at 30 °C for 5 min and for complete cleavage at 50 °C for 5 min, respectively. The reaction was terminated by adding 500 μL of sodium acetate solution (40 mM). For the identification of the phlorogucinol adducts, that is, (+)-catechin-(4α→2)-phloroglucinol ((+)-C-ph) and (−)-epicatechin-(4β→2)-phloroglucinol ((−)-EC-ph), authentic references were obtained by HSCCC according to published data (*29*). Phloroglucinol adducts were analyzed by reversed-phase HPLC-PDA (see above).

Nuclear Magnetic Resonance (NMR) Spectroscopy. One-dimensional ^{1}H NMR and two-dimensional ^{1}H−^{1}H correlation spectroscopy (COSY), ^{1}H−^{1}H total correlation spectroscopy (TOCSY), *J*-resolved spectroscopy, and rotating frame nuclear Overhauser enhancement spectroscopy (ROESY) experiments were acquired at low temperature, that is, 240 and 260 K, using a Bruker ARX400 NMR spectrometer (Rheinstetten, Germany) locked to the deuterium resonance of the solvent, acetone-d_6. Chemical shifts are given in parts per million (δ) relative to the residual proton signals of the solvent (2.05 ppm), and coupling constants are given in hertz.

Circular Dichroism. CD spectra were measured in methanol (ca. 1 mg/10 mL) using a Jasco J-715 CD spectropolarimeter (Gross-Umstadt, Germany). Scan parameters: bandwidth, 1.0 nm; sensitivity, 100 mdeg; response, 1 s; scan speed, 50 nm/min; step resolution, 0.1 nm.

RESULTS AND DISCUSSION

In previous studies (*10*, *11*), we have described the semisynthetic method in detail that has been used for the formation of six procyanidin trimers from a black chokeberry polymeric fraction (compounds **1**−**6**) and two procyanidin dimers (compounds **9** and **10**) from a white willow bark polymeric fraction. Trimers **1**−**6** contain (−)-epicatechin in the upper units, whereas in dimers (**9**, **10**) the upper unit consists of (+)-catechin. Procyanidin trimers with (−)-epicatechin and (+)-catechin in the upper and middle units were obtained by semisynthesis from a polymeric fraction of hazelnut seed coat fraction (compounds **7** and **8**). The chemical structures of the procyanidin trimers and dimers are shown in **Figure 1**. The isolation of compounds **1**−**10** was achieved by HSCCC (*10*, *11*) followed by a final purification by preparative HPLC. All compounds were obtained as pale white amorphous powders. In HPLC-ESI/MS/MS, all of the trimers **1**−**8** showed a molecular ion $[M - H]^-$ at *m*/*z* 865 (fragmentations at *m*/*z* 847, 739, 713, 695, 577, 575, 451, 425, 407, 289, 287, and 245), indicating a procyanidin skeleton composed of three (+)-catechin/(−)-epicatechin moieties, whereas dimers (**9** and **10**) gave a molecular ion $[M - H]^-$ at *m*/*z* 577 (fragmentations at *m*/*z* 559, 451, 425, 407, 289, 287, and 245), indicating two (+)-catechin/(−)-epicatechin moieties.

Structure Elucidation of Procyanidin Trimers. Six procyanidin trimers **1**−**6** were isolated and identified in the semisynthesis from a polymeric *A. melanocarpa* fraction. The 1D ^{1}H NMR data are shown in **Table 1**.

The 1D ^{1}H NMR spectra of **1** in acetone-d_6 at various temperatures are depicted in **Figure 2**. At ambient temperature (300 K) the spectra consists of a series of broad and sharp lines caused by conformational phenomena associated with hindered rotation. Previously such compounds have been converted into their peracetates or methyl ether acetates to yield sharp spectra, and ^{1}H NMR spectra were then measured at high temperature (*14*−*17*, *22*). In our studies, however, a set of sharp ^{1}H NMR signals belonging to one major molecular species was obtained in acetone-d_6 as solvent by lowering the temperature to 240 K. Integration and inspection of the changes in chemical shift upon lowering of the temperature allow identification of the hydroxyl protons that were confirmed by H/D exchange with methanol-d_4. The nature of the units present in **1**−**10** follows from the magnitude of the vicinal coupling constants of the C-ring protons after their unambiguous assignment. A large value of 8−10 Hz for $^3J_{2,3}$ indicates a (+)-catechin unit (2,3-*trans*) and, in general, ~2 Hz or a broad singlet an (−)-epicatechin unit (2,3-*cis*). The signal widths and observable couplings $^3J_{2,3}$ and $^3J_{3,4}$ in **1** were small in magnitude, indicating gauche dihedral angles compatible with the presence of three (−)-epicatechin units.

Signals of the carbon-bound protons of the three (−)-epicatechin units were found in the region δ_H 6.6−7.2 corresponding to H2′,

Table 1. ^{1}H NMR Spectral Data of Procyanidin Trimers **1**−**6** in Acetone-d_6 at 240 K

		δ multiplicity (J)					
ring	position	trimer **1**	trimer **2**	trimer **3**	trimer **4**[d]	trimer **5**	trimer **6**
				Upper Unit			
A	H6/H8	5.98 d (2.0)[a]	5.98 d (2.3)[a]	5.95 d (2.2)[a]	H8: 6.01 d (2.2)	6.01 d (1.9)[a]	6.01 d (2.0)[a]
A	H6/H8	6.01 d (2.1)[a]	6.00 d (2.3)[a]	6.08 d (2.3)[a]	H6: 6.13 d (2.0)	6.05 d (2.2)[a]	6.06 d (2.0)[a]
B	H2′	6.95 d (1.8)	6.95 d (2.0)	7.01 d (1.6)	6.94[e] d (1.8)	7.03 d (2.0)	7.05 d (2.0)
B	H5′	6.70 d (8.0)	6.70 d (8.1)	6.70 d (8.1)	6.72 d (8.1)	6.74 d (8.2)	6.73 d (8.1)
B	H6′	6.61 dd (2.0, 8.1)	6.60 dd (2.0, 8.2)	6.72[b]	6.69−6.65	6.67[b]	6.64 dd (1.9, 8.4)
C	H2	5.07 s_{br}	5.05 s_{br}	4.83 s_{br}	4.91 s_{br}	5.03 s_{br}	5.02 s_{br}[g]
C	H3	4.02 m	4.04 m	3.84 m	3.82 m	4.02 m	4.01 m
C	OH3[d]	4.27−4.30	4.26	4.48	4.24	4.36	4.36
C	H4	4.78 d (2.0)	4.78 s_{br}	4.62 s_{br}	4.46 d (∼2.0)	4.81 d (2.0)	4.81 s_{br}
				Middle Unit			
A	H6/H8	H6: 5.92 s	H6: 5.91 s	H8:[c] 5.93 s	H8:[a] 5.92 s	H6:[c] 6.05 s	H6:[c] 6.03 s
A	OH7	7.10	7.02			7.10[f]	7.34
B	H2′	7.07 d (2.0)	7.10 d (1.9)	6.98 d (2.0)	7.00 (6.97[e]) d (1.8)	7.07 d (1.6)	7.06 d (1.6)
B	H5′	6.65 d (8.2)	6.66 d (8.4)	6.69 d (8.1)	6.72 d (8.1)	6.76 d (8.2)	6.69−6.79
B	H6′	6.71[b]	6.77 dd (2.0, 8.3)	6.65 dd (2.0, 8.2)	6.65−6.69	6.69[b]	6.72[b]
C	H2	5.20 s_{br}	5.25 s_{br}	4.88 s_{br}	4.79 s_{br}	5.03 s_{br}	5.03 s_{br}[g]
C	H3	4.07 m	4.08 m	3.89 m	3.87 m	4.19 m	4.19 m
C	OH3[d]	4.27−4.30	4.21	4.32	4.53	4.49	4.49
C	H4	4.81 d (2.2)	4.78 s_{br}	4.55 s_{br}	4.60 d (2.4)	4.74 d (2.0)	4.74 s_{br}
				Terminal Unit			
A	H6/H8	H6: 5.94 s	H6: 5.91 s	H6:[c] 6.14 s	H6:[a] 6.10 s	H8:[c] 6.07 s	H8:[c] 6.04 s
A	OH7	7.20	7.23			7.36[f]	
B	H2′	7.18 d (1.7)	6.90 d (2.0)	7.04 d (2.1)	6.78 d (2.1)	7.06 d (2.0)	6.87 d (1.8)
B	H5′	6.71 d (8.1)	6.69 d (8.3)	6.61 d (8.2)	6.56 d (8.1)	6.77 d (8.2)	6.69−6.79
B	H6′	6.92 dd (1.7, 8.2)	6.89 dd (2.0, 8.3)	6.81 dd (2.1, 8.3)	6.69−6.65	6.82 dd (2.0, 8.2)	6.75 dd (1.8, 8.1)
C	H2	5.07 s_{br}	4.90 d (5.9)	4.91 s_{br}	4.61 d (6.7)	4.83 s_{br}	4.48 d[b]
C	H3	4.36 m	4.08 m	4.24 m	3.92 m	4.13 m	3.96 m
C	OH3[d]	4.27−4.30	4.56	3.91	4.44	4.19	4.47
C	H4A	2.89 dd (4.4, 16.4)	2.64 dd (5.0, 16.4)	2.83 dd (4.7, 16.9)	2.69 dd (5.4, 16.4)	2.81 dd (4.2, 17.1)	2.84 dd (5.4, 16.1)
C	H4B	2.70 dd (<1, 16.5)	2.57 dd (6.3, 16.3)	2.63 dd (<1, 16.9)	2.47 dd (7.7, 16.3)	2.69 dd (<1, 16.9)	2.44 dd (9.1, 16.1)

[a] Interchangeable. [b] Overlap prevents determination of coupling constants. [c] Interchangeable. [d] Specific assignments of the OHs bound to C3 of the various units follow from the correlation to H3 in the TOCSY spectrum. The aromatic OH signals of **4** showed sharp signals whose assignments follow from correlations in the 2D ROESY spectrum. These were observed for the upper unit at 9.44 (OH5), 9.17 (OH7), 8.41 (OH3′), and 8.39 (OH4′), for the middle unit at 9.44 (OH5), 8.68 (OH7), 8.47 (OH3′), and 8.47 (OH4′), and for the terminal unit at 9.24 (OH5), 9.26 (OH7), 8.39 (OH3′), and 8.36 (OH4′). [e] After H/D exchange with a small amount of CD_3OD. [f] Interchangeable. [g] Interchangeable.

H5′, and H6′ of the three catechol B-rings (∑ 9 protons), δ_H 5.9−6.0 to H6 and H8 of the three A-rings (∑ 4 protons), δ_H 4.0−5.2 to H2, H3, and H4 of the three heterocyclic C-rings (∑ 8 protons), and the two geminal H4 protons of the terminal unit at δ_H 2.7−2.9 (H4A, δ_H 2.89, dd, $^3J_{3,4}$ 4.4, 16.4; H4B, δ_H 2.70, dd, $^3J_{3,4}$ < 1, 16.5).

Specific assignments were then made from consideration of 2D ^{1}H COSY, TOCSY, and ROESY spectra recorded at low temperature (**Table 1**). Characteristic couplings distinguished H6 and H8 of the upper unit at δ_H 5.98 and 6.01 from H6 of the middle and terminal units, as well as allowed immediate identification of H4A and H4B of the terminal unit. COSY and TOCSY spectra then enabled identification of H2, H3, and OH3 of the latter unit, as well as of the corresponding signals in the upper and middle units. The same spectra afforded identification of the B-ring three-proton spin systems (Figure 3 in the Supporting Information). Long-range correlations of H2′ and H6′ of the B-rings identified the H2 signals in the COSY spectrum.

Unambiguous identification of the individual B- and C-ring protons then followed from the through-space correlations observed in the ROESY spectra. Thus, NOE correlations of aromatic protons H2′ and H6′ of the B-ring with H2, H3, and OH3 of ring C allowed assignment of these signals in the B- and C-rings of the terminal unit. The same correlations allowed identification of H2 and H3 in the upper and middle units, which cannot be distinguished. However, an additional NOE correlation of H2′ and H6′ of the middle and terminal units with H4 of the upper and middle units allowed the unambiguous differentiation of both units (**Figure 4**). Such an interaction has been demonstrated for flavanol−anthocyanin dimers (*24*) and also for the procyanidin dimers (*11*), being characteristic of a 4→8 interflavanoid linkage. In addition, two high-field aromatic OHs at δ_H 7.1 (middle unit) and 7.2 (terminal unit) showed NOEs to the adjacent H6 and an additional correlation with H2 C-ring of the upper and middle units, respectively (**Figure 4**). The exact assignment of the 10 low-field aromatic OHs (δ_H 8.1−8.8) is not possible. The three aliphatic OHs of the C-rings overlap in the region δ_H 4.27−4.30.

Thus, by using a combination of 2D through-bond and through-space correlations it is possible to unambiguously assign all of the carbon-bound protons in compound **1** (Figure 5 in the Supporting Information).

As we have noted above, the 4→8 interflavanoid bond is defined by interactions of B-ring protons with H4 of an adjacent system that were present in the ROESY spectrum. This spectrum also showed a correlation of H2 with H4A of the terminal unit, but no correlations between H2 and H4 in the upper and middle units. In these units H4 correlates with OH3, thus indicating that both 4→8 interflavanoid bonds have a β configuration.

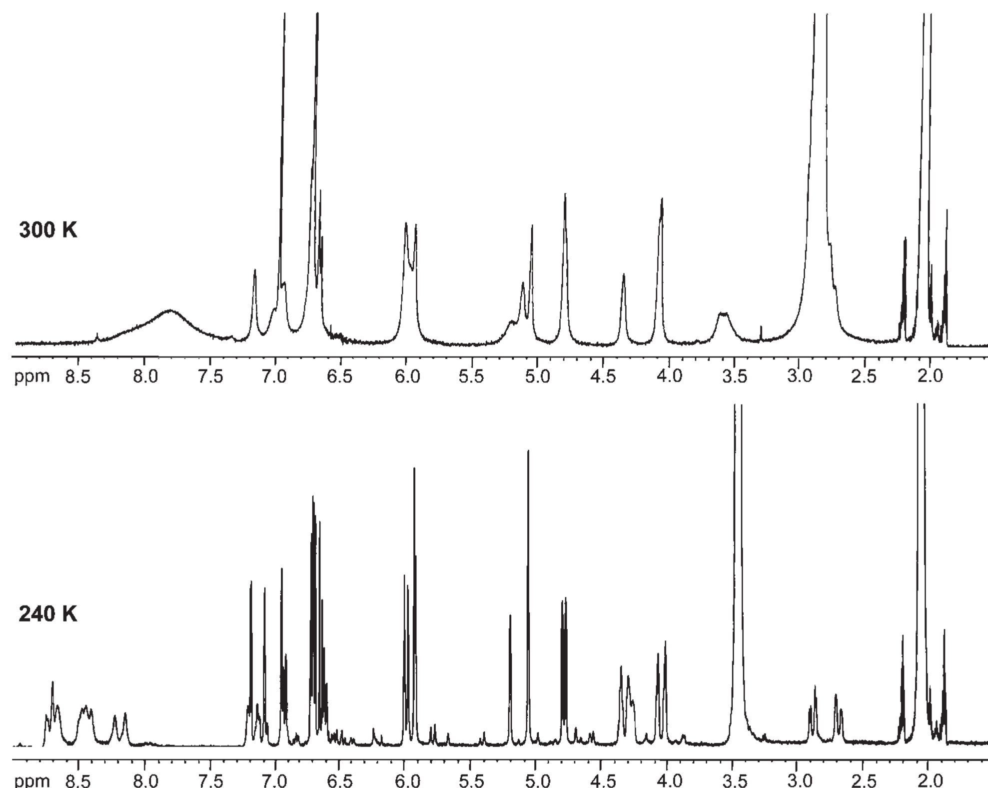

Figure 2. Effect of lowering the temperature from 300 to 240 K on the 1D ^{1}H NMR spectrum of **1** in acetone-d_6.

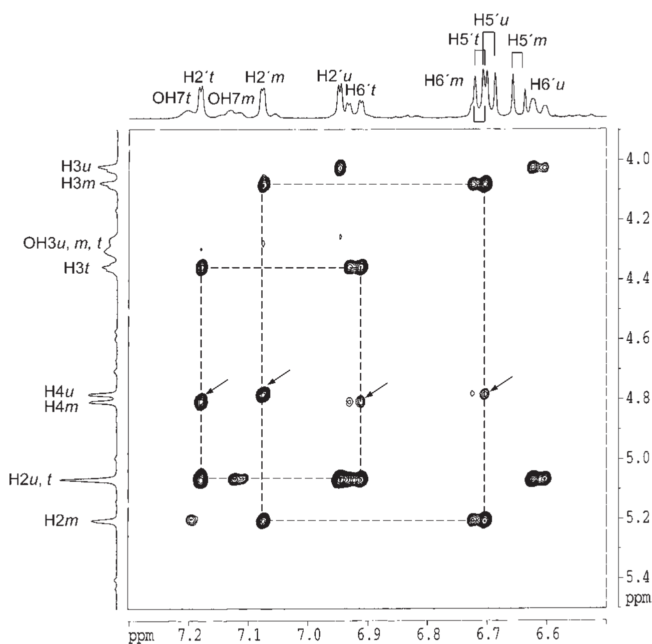

Figure 4. 2D ROESY spectrum of **1** at 240 K used in the assignment of the interflavanoid bond, where the upper, middle, and terminal units are indicated as *u*, *m*, and *t*, respectively. Note the interunit correlations, marked with arrows, defining the 4→8 interflavanoid linkages.

Proanthocyanidin oligomers often exist as rotamers in solution, and duplication of NMR signals has been attributed to conformers resulting from a rotation around the interflavonoid bond. In the present case exchange signals observed in the ROESY spectrum of **1** indicate all of the small signals are a result of the presence of higher energy conformations of the molecule (not impurities). Inspection of the region δ_H 4.5−5.5 shows the signals of H2 and H4 of the upper and middle units each correlate with a number of smaller signals, suggesting an equilibrium mixture containing a least two further species (Figure 6 in the Supporting Information). Thus, trimer **1** (−)-epicatechin-4β→8-(−)-epicatechin-4β→8-(−)-epicatechin ((−)-EC-4β→8-(−)-EC-4β→8-(−)-EC) is present in solution at low temperature in one major conformation and at least two minor conformations due to restricted rotation about both interflavanoid bonds.

Confirmation of the structures of the procyanidins **1**−**10** was furnished by acid-catalyzed degradation (phloroglucinolysis). The cleavage of the interflavanoid bonds releases the extension units as carbocations, which reacted with the nucleophilic reagent (phloroglucinol) to form stable flavan-3-ol-phloroglucinol adducts (*28*). The terminal units are obtained as the corresponding flavan-3-ols. These cleavage products were then identified by reversed-phase HPLC-PDA and ESI/MS analysis. Full details are given in the Supporting Information.

A similar NMR approach as exemplified for trimer **1** has been used to identify compounds **2**−**10**, and only the pertinent points for the assignment of the remaining compounds will be given here. The ^{1}H NMR data of compound **2** (**Table 1**) indicate that the upper and middle units of **2** are the same as in trimer **1**. In **2** the geminal H4 protons show double doublets at δ_H 2.57 ($^3J_{3,4}$ 6.3, 16.3) and δ_H 2.64 ($^3J_{3,4}$ 5.0, 16.4), and H2 shows a doublet ($^3J_{2,3}$ 5.9) at δ_H 4.90. The larger values observed for both $^3J_{2,3}$ and $^3J_{3,4}$ of the terminal unit of **2** compared to **1** are indicative of the heterocyclic C-ring of (+)-catechin in a distorted half-chair geometry and reflect the conformational flexibility of this system compared to the (−)-epicatechin system found in **1** (*19*). Hence, the structure of **2** could be assigned as (−)-epicatechin-4β→8-(−)-epicatechin-4β→8-(+)-catechin ((−)-EC-4β→8-(−)-EC-4β→8-(+)-C), which was confirmed by phloroglucinolysis.

Compound **3** consisted of three (−)-epicatechin units. ^{1}H NMR data of **3** in acetone-d_6 are shown in **Table 1**, and the assignments of the interflavanoid linkages were deduced from the

NOE interactions. The ROESY spectrum showed no interaction between H2′ and H6′ of the middle unit and H4 of the upper unit, whereas H2′ and H6′ of the terminal unit interacted with H4 of the middle unit. Hence, the structure of **3** is (−)-epicatechin-4β→6-(−)-epicatechin-4β→8-(−)-epicatechin ((−)-EC-4β→6-(−)-EC-4β→8-(−)-EC).

The upper and middle units of compound **4** consisted of (−)-epicatechin, and the terminal unit was (+)-catechin as in trimer **2** (^{1}H NMR data in **Table 1**). The overlapped signals, H4 of the middle unit with H2 of the terminal unit, prevented a direct assessment of H2′ and H6′ of the terminal unit with H4 of the middle unit. However, at low temperature the spectra of **4** showed 12 well-resolved aromatic OH groups that could be assigned from their NOE interactions in the ROESY spectrum. H4 of the middle unit interacted with OH5 of the middle and OH7 of the terminal unit. The latter, together with OH5, interacted with H6 of the terminal unit, which was unambiguously assigned, indicating a 4→8 linkage. Similarly, H8 was assigned from its interaction with OH7 of the middle unit, suggesting a 4→6 linkage for the upper and middle unit. This assignment was confirmed by phloroglucinolysis. Hence, **4** was assigned as (−)-epicatechin-4β→6-(−)-epicatechin-4β→8-(+)-catechin ((−)-EC-4β→6-(−)-EC-4β→8-(+)-C).

From the ^{1}H NMR data of compounds **5** and **6** (**Table 1**) and comparable analyses as presented above, the chemical structures were elucidated as (−)-epicatechin-4β→8-(−)-epicatechin-4β→6-(−)-epicatechin ((−)-EC-4β→8-(−)-EC-4β→6-(−)-EC) and (−)-epicatechin-4β→8-(−)-epicatechin-4β→6-(+)-catechin ((−)-EC-4β→8-(−)-EC-4β→6-(+)-C), respectively. The structures were confirmed by phloroglucinolysis.

In 1985 Hsu et al. (*30*) analyzed the structures of (−)-epicatechin-4β→8-(+)-catechin-4α→8-(−)-epicatechin (**7**) and (−)-epicatechin-4β→8-(+)-catechin-4α→8-(+)-catechin (**8**). The ^{1}H NMR spectrum of **7** was complicated by conformational isomerism (*12*, *13*) and provided no structural information, although the appearance of six major peaks, in ^{13}C NMR spectra, attributable to flavans C2 and C3, confirmed its triflavanoid constitution (*30*). The ^{13}C NMR shifts indicated the presence of (+)-catechin and (−)-epicatechin, but a definitive structural assignment of **7** required the acid-catalyzed degradation with benzylmercaptan, and the structure of **8** was determined only by thiolytic degradation (*30*). Later, Foo and Karchesy determined the structure of **8** with the same arguments (^{13}C NMR and degradation) as described in Hsu et al. (*14*, *30*). Saito et al. carried out ^{1}H and ^{13}C NMR measurements of trimers **1**, **2**, **7**, and **8** after peracetylation. However, an exact assignment of the resulting data was not attempted (*22*). More recently, an assignment of the peracetyl derivative of **8** has been reported from which the interflavanoid linkage was indirectly determined using HMBC and chemical shift data (*31*).

In the spectra of **7** and **8** the presence of (+)-catechin in the middle unit leads to hindered rotation around the interflavanoid bonds, producing equally populated rotamers. Hence, the ^{1}H NMR spectrum of **7** at 240 K is extremely complex, and exchange peaks in the ROESY spectrum indicate the presence of two major conformers in a 1:1 ratio. The assignment of all signals was possible for both conformers from a combination of the COSY data before exchange and the ROESY data after H/D exchange (**Table 2**). The magnitude of the coupling constants in the C-ring, particularly those to H2, immediately identified the nature of the three units as (−)-epicatechin in the upper and terminal units and (+)-catechin in the middle unit. The large coupling between H3 and H4 of this latter system indicates the equatorial disposition of the terminal unit, which was confirmed from the observation of an interaction between H2 and H4 of the middle unit in the ROESY spectrum. A similar interaction was not found between H2 and H4 of the upper system, thus indicating an equatorial disposition of H4 in the upper C-ring. The two interflavonoid bonds were evident, as in **1**, from the ROESY spectrum after H/D exchange. These data combined with phloroglucinolysis allowed unambiguous identification of **7** as (−)-epicatechin-4β→8-(+)-catechin-4α→8-(−)-epicatechin ((−)-EC-4β→8-(+)-C-4α→8-(−)-EC).

Table 2. ^{1}H NMR Spectral Data of **7** in Acetone-d_6 with a Trace of CD_3OD at 240 K

ring	position	δ multiplicity (*J*) rotamer A (∼50%)	rotamer B (∼50%)
		Upper Unit	
A	H6/H8[a]	5.27 d (2.3) + 5.65 d (2.3)	
A	H6/H8[a]	5.87 d (2.3) + 5.89 d (2.3)	
B	H2′	6.90 d (1.7)	6.97 d (∼1.7)
B	H5′	6.63−6.72	
B	H6′	6.63−6.72	
C	H2	5.38 s_{br}	5.09 s_{br}
C	H3	3.73 m	3.83 m
C	H4	4.52 d (2.0)	4.59 d (2.0)
		Middle Unit	
A	H6[b]	5.75 s + 5.94 s	
B	H2′	6.62 d (2.0)	7.02 d (2.0)
B	H5′	6.56 d (8.0)	6.67 d (7.1)
B	H6′	6.39 dd (2.0, 8.1)	6.90 dd (1.9, 7.2)
C	H2	3.85 d (10.0)	4.52 d (9.8)
C	H3	4.40 m	4.65 m
C	H4	4.62 d (7.4)	4.78 d (7.6)
		Terminal Unit	
A	H6[b]	5.98 s + 6.02 s	
B	H2′	7.14 d (2.0) + 7.16 (2.0)	
B	H5′	6.70 d (8.1) + 6.73 (8.1)	
B	H6′	6.76 dd (∼2.0, 8.3) + 6.78 dd (2.0, 8.2)	
C	H2	4.98 s_{br} + 4.99 s_{br}	
C	H3	4.27 m	
C	H4B	2.85 dd (<1, 16.6)	
C	H4A	2.91 dd (4.2, 16.9)	

[a] Interchangeable. [b] Interchangeable.

The spectra of compound **8** showed a further order of complexity. In the δ_H 6.5−3.5 region of the 1D ^{1}H spectrum at 240 K after H/D exchange with a small amount of CD_3OD, several nonexchangeable protons appeared four times in the spectra and showed a complex set of exchange signals. Thus, in the 2D COSY, ring A of the upper unit showed four systems of two coupled protons (H6 with H8, $J \sim 2$ Hz) in which each proton showed an exchange peak with a second system, suggesting the presence of four conformers arising from the presence of two (+)-catechin units. As expected, there were two sets of overlapping signals centered at δ_H 2.88 and 2.51 that showed characteristic multiplets of H4A and H4B of a (+)-catechin system, indicating the latter as the terminal unit. In the region δ_H 5.4−3.5 of the 2D COSY spectrum, there were four AMX systems (H2/H3/H4) with small vicinal coupling constants indicative of an (−)-epicatechin system. The complexity of the spectra prevented a more detailed analysis of **8**. Hence, the data combined with phloroglucinolysis confirmed **8** as (−)-epicatechin-4β→8-(+)-catechin-4α→8-(+)-catechin ((−)-EC-4β→8-(+)-C-4α→8-(+)-C).

Structure Elucidation of Procyanidin Dimers B6 and B8. The occurrence of procyanidin dimers B6 (**9**) and B8 (**10**), differing only in the nature of the terminal unit, is rare in nature as C8 in ring A is more nucleophilic and less sterically hindered than C6. Semisynthesis and fractionation by HSCCC (*11*), followed by

final purification by preparative HPLC, have recently been reported by us. The structures of dimers **9** and **10** have been elucidated using the same approach as used above for the procyanidin trimers. Again, complex ^{1}H NMR spectra arising from rotational isomerism were observed due to the presence of (+)-catechin in the upper unit. At low temperature these showed nearly equal amounts of two rotamers for both B6 (47 and 53%) and B8 (~50%). Further clarification of the structure followed from the ^{1}H NMR data, and phloroglucinolysis (*11*) indicated **9** was procyanidin dimer B6 ((+)-catechin-4α→6-(+)-catechin ((+)-C-4α→6-(+)-C)) and **10** B8 ((+)-catechin-4α→6-(−)-epicatechin ((+)-C-4α→6-(−)-EC).

Circular Dichroism. The absolute configuration at C4 of the interflavanoid linkage was assigned from CD spectroscopic data. Positive Cotton effects at 220−240 nm indicated the β-configuration for compounds **1**−**6** and negative Cotton effects at 220−240 nm α-configuration for compounds **9** and **10**. These results are in agreement with literature data (*25*, *32*, *33*). The absolute configuration of compounds **7** and **8** could not be unambiguously determined from the observed negative Cotton effects, although these were similar to those of (+)-C-4α→8-(−)-EC-4β→8-(−)-EC (*15*).

In summary, the direct analysis of procyanidin dimers and trimers using 1D and 2D ^{1}H NMR techniques at low temperatures with acetone-d_6 as solvent enable an unambiguous structure elucidation without the necessity of derivatization. Rotational isomerism usually causes signal broadening at ambient temperature. At low temperature these signals are well resolved, allowing complete assignment of the major rotamer(s). Previously, the connection of the interflavanoid bond in procyanidins has been determined from the observation of heteronuclear long-range ^{1}H−^{13}C interactions in 2D HMBC spectra and the recorded chemical shifts (*17*, *19*, *21*, *33*−*35*). In the present paper, ROESY spectra clearly show the configuration at C4 (α or β) and provide a spectroscopic means of distinguishing a 4→8- from a 4→6-linked procyanidin dimer or trimer without the need for derivatization. This new method requires only small amounts of sample and avoids the use of time-consuming 2D heteronuclear NMR measurements. Phlorogucinolysis provides confirmation of the NMR analyses. In the case of all 4→8-connected trimers, mild degradation by phloroglucinolysis results in traces (< 1%) of unreacted trimer, whereas in the case of mixed 4→8/4→6-connected trimers 10−19% unreacted trimer was found. These differences allow the fast and accurate determination of the interflavanoid bond with only a small amount of isolated sample material (~0.1 mg).

ACKNOWLEDGMENT

We thank Christel Kakoschke (HZI) for technical support.

Supporting Information Available: Phloroglucinolysis data and Figures 3, 5, and 6. This material is available free of charge via the Internet at http://pubs.acs.org.

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Received for review August 20, 2010. Revised manuscript received November 19, 2010. Accepted November 20, 2010. We thank the German Federal Ministry of Education and Research "BMBF" (Grant 0313828C) for financial support.