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PROCYANIDIN TRIMERS POSSESSING A DOUBLY LINKED STRUCTURE FROM *AESCULUS HIPPOCASTANUM*

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Key Word Index—*Aesculus hippocastanum*; Hippocastanaceae; fruit shells; proanthocyanidins; A-types; trimers; structure elucidation; chemical degradation.

Abstract—Following tentative HPLC peak identification by UV/CRD absorbance ratios, structural examination of the phenolic metabolites of the fruit shells of *Aesculus hippocastanum* has led to the isolation and characterization of the uncommon procyanidin trimers, epicatechin-(4β,8;2β,7)-catechin-(4β,8)-epicatechin, epicatechin-(4α,8)-epicatechin-(4β,8;2β,7)-epicatechin, epicatechin-(4β,8)-epicatechin-(4β,8;2β,7)-epicatechin and epicatechin-(4β,8;2β,7)-epicatechin-(4α,8)-*ent*-epicatechin, possessing doubly linked units, associated with epicatechin-(4β,6)-epicatechin-(4β,6)-epicatechin. Amongst these, the first two mentioned compounds represent novel natural metabolites. The structures were determined by analysis of spectral data (^{1}HNMR, FAB-mass spectrometry, CD) and chemical degradation.

INTRODUCTION

The increasing interest in proanthocyanidins, widely distributed in the plant kingdom, is based on a variety of biological activities. The extreme complexity of tannin extract compositions and the close structural similarity of constituent oligomeric proanthocyanidins represent severe complicating factors in the characterization of oligomeric flavan-3-ols of many traditionally used polyphenolic plants. In order to facilitate initial identification of proanthocyanidin patterns of plant extracts, and hence assisting in time-saving screenings for interesting metabolites, we have recently introduced a new HPLC method in which peaks are analysed by on-line UV and chemical reaction detection, resulting in characteristic absorbance ratios for individual proanthocyanidins [1]. By applying this methodology to extracts from *Aesculus hippocastanum*, known to contain a wealth of A- and B-type proanthocyanidins [2–4], the appearance of relevant peaks in the HPLC chromatograms as indicated by the UV/CRD absorption ratio prompted the isolation and structural elucidation of the corresponding compounds.

RESULTS AND DISCUSSION

Structural examination of the phenolic extractives of the seed shells of *A. hippocastanum*, selected by the HPLC peak detection method [1], revealed additional doubly linked proanthocyanidins based upon 3,3′,4′,5,7-pentaoxygenated monomeric precursors, not previously encountered in this natural source. Amongst these, epicatechin-(4β,8;2β,7)-catechin-(4β,8)-epicatechin (**3**) and epicatechin-(4α,8)-epicatechin-(4β,8;2β,7)-epicatechin, (**7**), represent novel metabolites of the above class of analogues possessing doubly- linked flavanyl units, while the natural occurrence of epicatechin-(4β,8)-epicatechin-(4β,8;2β,7)-epicatechin (**5**), a stereoisomer of **7**, and the remaining trimeric proanthocyanidins **1** and **9** has only recently been documented.

Their 300 MHz ^{1}H NMR spectra, plagued by the adverse effects of dynamic rotational isomerism, were in parts exceedingly complex, thus rendering their interpretation extremely difficult. Structural assessment of all compounds was therefore effected by a combination of chemical degradation and spectroscopic evidence. Acid hydrolysis of each compound produced cyanidin, consistent with the anticipated 3′,4′-*ortho*-dihydroxylation of the B-ring in each instance. Supporting evidence regarding the presence of an A-type flavanyl unit in **5** and **7** was obtained from partial acid-catalysed degradation yielding epicatechin-(4β,8;2β,7)-epicatechin (**14**), identified by co-chromatography with an authentic specimen.

The FAB-mass spectrum of **1** indicated a $[M + H]^+$ ion at *m/z* 865, corresponding to a triflavanoid structure. Although the ^{1}H NMR spectra of the acetate (**2**) at ambient temperatures exhibited line-broadening and duplication of resonances, phenomena indicative of intermittent

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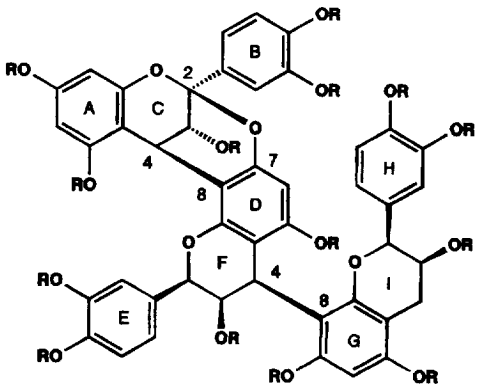

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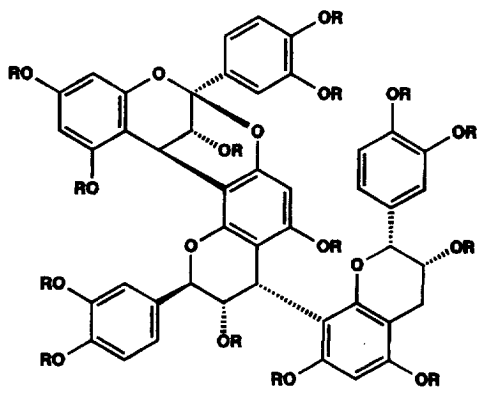

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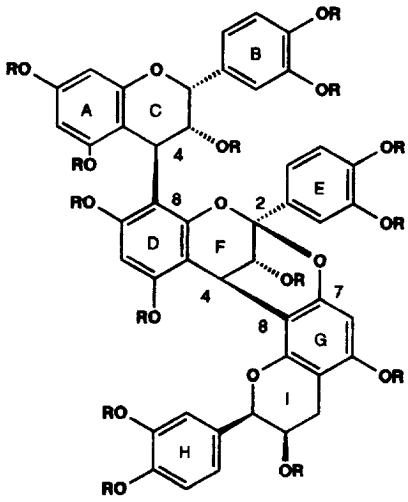

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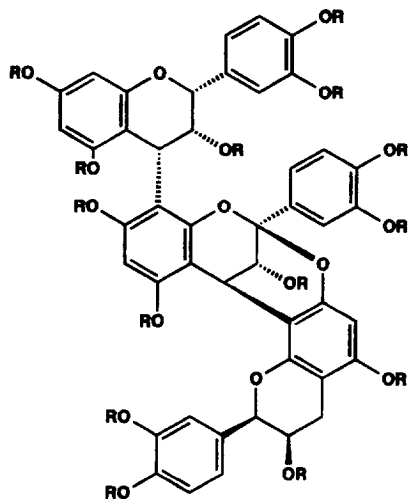
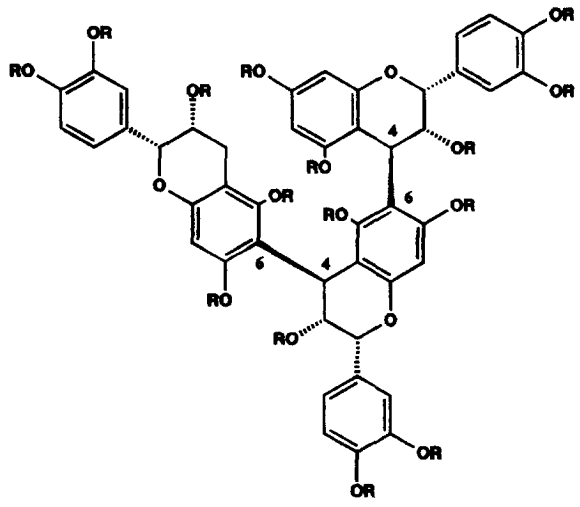


7 R = H

8 R = Ac

exchange of rotational isomers, the well-resolved resonances permitted not only the allocation of protons, but also the differentiation of rotameric signals by ^1H–^1H correlation spectra. The spin systems thus detected the chemical shifts of aromatic and heterocyclic protons, and the coupling constants were reminiscent of epicatechin-(4β,8;2β,7)-epicatechin-(4α,8)-*ent*-epicatechin, the natural existence of which has only very recently been demonstrated by its isolation from *Pavetta owariensis* [5]. The identity was also indicated by a very similar strong positive Cotton effect at 237 nm in the CD curve of **2**, reflecting a 4β-flavanyl unit as depicted in the structure between the 'upper' (ABC) and the 'middle' (DEF) constituent units. As the spectroscopic data of **2**, being reported from a plant source for the second time, have already extensively been discussed [5, 6], this will not be repeated here.

In the FAB-mass spectrum of **3**, a $[M + H]^+$ ion was detected at *m/z* 865, which suggested a triflavanoid consti-



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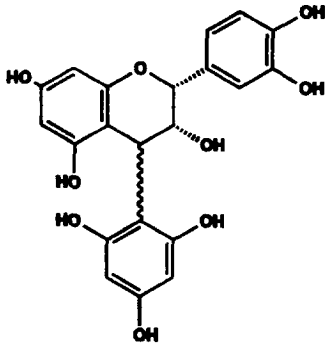

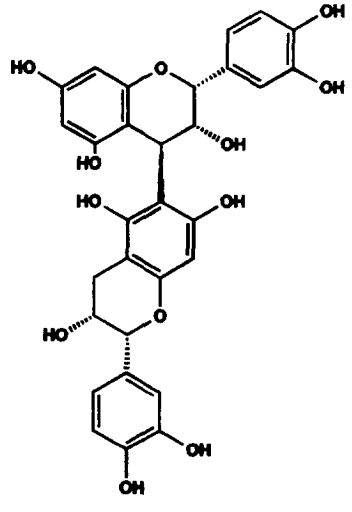

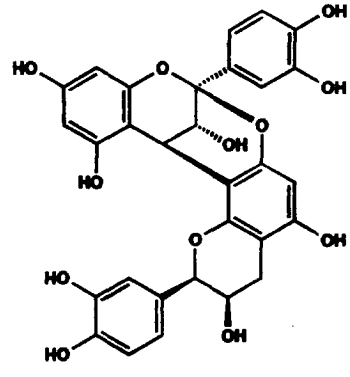

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tution. Its ^{1}H NMR spectrum at 300 MHz in $(CD_3)_2CO$ displayed the characteristic AB-system [δ3.45, 3.97, both *d*, J = 3.5 Hz, 3- and 4-H(C), respectively] associated with A-type proanthocyanidins. Again, at room temperature, the ^{1}H NMR spectrum revealed the familiar presence of the effects of dynamic rotational isomerism and indicated two predominant rotameric forms, the relative populations of the two states being *ca* 3 : 1 as judged from signal intensities. The conspicuous broadening of heterocyclic proton resonances was reminiscent of those prominent signals which characterize spectra of 2,3-*cis* oligoflavanoids.

Owing to overlap of signals in the ^{1}H NMR spectrum of the free phenol, structural assessment of **3** was effected by preparing its acetate (**4**) as a suitable derivative for the successful application of a series of spin–spin decoupling experiments. Thus, irradiation of the broadened signal at δ2.90, readily attributable to the methylene functionality in ring I, led to selective sharpening of the broad absorption at δ5.37 and, hence, detection of H-3(I). Correlation of this

signal with the H-2 singlet at δ4.88 via a spin decoupling experiment defined the heterocyclic protons of the I-ring. Allocation of the remaining heterocyclic F-ring protons was effected by decoupling procedures using the H-3(F) multiplet (δ5.84), characteristic of a catechin entity, as reference signal. Coupling constants reflected the uncommon 2,3-*trans*-3,4-*cis* configuration for ring F ($J_{2,3}$ = 10.0 Hz, $J_{3,4}$ = *ca* 6.0 Hz, partially overlapped by rotameric signals) and familiar 2,3-*cis* (J < 1.0 Hz) for the I-ring [7].

Further evidence for the sequence of constituent units of **3** was obtained by partial acid-catalysed cleavage. The generation of epicatechin confirmed the identity of the 'lower' terminal unit, as already concluded from coupling constants of heterocyclic protons of the I ring.

Although the (4,8)-interflavanyl linkage between the terminal epicatechin unit and the A-type extension component was already indicated by the rotamer population as cited above [8], definition of the mode of coupling followed from the chemical shifts of H-2 and H-3 resonances of the 'lower' epicatechin unit. The relatively upfield position of H-2(I) (δ4.88) and the enhanced chemical shift difference [$\Delta\delta$0.49 ppm vs 0.21 ppm for the analogous biflavanoid derivative of catechin-(4α,8)-epicatechin] between H-2(I) and H-3(I) correlates with similar observations, associated with (2*R*,3*S*,4*R*)-2,3-*trans*-3,4-*cis*-procyanidin units as the 8-substituent [7, 9]. Examination of Dreiding models also showed greater steric hindrance between flavanyl units for an analogous 3,4-*trans* arrangement. When considered in conjunction with the coupling constants of heterocyclic F-ring protons, indicative of a 2,3-*trans*-3,4-*cis* configuration of the heterocycle (*vide supra*), the strong positive Cotton effect at 228 nm in the CD spectrum of **4** also reflected the (2β,4β)-orientation and, hence, the absolute configuration depicted in its formulation. Finally, the weak but selective ^{1}H NOE association of H-3(C) to H-6(D) (δ6.61) defined the relative 3,4-*trans* stereochemistry of the C-ring [10]. Collectively these data identified **3** as epicatechin-(4β,8;2β,7)-catechin-(4β,8)-epicatechin, a new natural product, structurally closely related to aesculitannin B [4], but differing in stereochemistry. In this context, it should be noted that aesculitannin B falls in the group of trimeric A-type proanthocyanidins, which we have recently drawn attention to with regard to inconsistencies between naming and graphical representation [11].

Two other proanthocyanidins, the trimers epicatechin-(4β,8)-epicatechin-(4β,8;2β,7)-epicatechin and epicatechin-(4α,8)-epicatechin-(4β,8;2β,7)-epicatechin, **5** and **7**, respectively, were found to contain A-type units as for **1** and **3**, but differing with regard to the location of the doubly linked flavanyl entity as evidenced by the products obtained from cleavage (*vide infra*). Owing to the complexity of ^{1}H NMR spectra of both free phenols and corresponding acetate derivatives, i.e. excessive line-broadening and overlap of signals presumably due to the effects of dynamic rotational isomerism, the structures of **5** and **7** could not be confirmed by spectroscopy, limiting their analysis primarily to acid-catalysed degradative studies.

Thus, treatment of **5** and **7** with 0.5 M ethanolic HCl generated epicatechin (4β,8;2β,7)-epicatechin, **14**, in each instance, identical to an authentic sample. Similar partial acid-catalysed degradation of each compound, but in the presence of phloroglucinol gave, in addition to the A-type cleavage product **14** as before (formed from the 'lower' terminal unit), epicatechin-(4β,2)-phloroglucinol (**11**) (derived from the extender unit) identified by HPLC analysis (R_t 10.4 min). The sequence of constituent flavanyl units in both **5** and **7** was therefore defined to be that of an epicatechin-epicatechin-(4β,8;2β,7)-epicatechin trimer, also immediately suggesting it to be differentiated by the mode of bonding between the 'upper' and 'middle' flavanyl unit, i.e. (4,8)- and (4,6)-couplings respectively. Unlike **5**, however, reaction of **7** with phloroglucinol furnished two phloroglucinol-containing adducts as indicated by peaks with R_t 10.4 (major) and 9.9 min (minor) in the HPLC chromatograms, differing from catechin-derived phloroglucinol analogues with regard to chromatographic properties. These observations cannot be explained on the basis of the supposed differing bonding points. Conjecture regarding the alternative of 4β and 4α orientations at C-4(C) found support in the CD spectra of **5** and **7**, displaying Cotton effects of opposite signs (positive and negative, respectively) at 215 nm, in complete agreement with their depicted structures. The generation of two phloroglucinol adducts may be rationalized on the basis of ready isomerization of the 3,4-*cis* product (**12**) to the thermodynamically more stable 3,4-*trans* analogue **11**, similar to their behaviour during thiolysis [12]. Owing to insufficient sample quantities, ^{1}H NMR analysis of the cleavage products and, hence, comparison with recently published data for 2,3-*cis*-3,4-*trans*- and 2,3-*cis*-3,4-*cis*-phloroglucinol adducts [13] was precluded. Upon consideration of the above results, **5** and **7** were characterized as epicatechin-(4β,8)-epicatechin-(4β,8;2β,7)-epicatechin, conforming with aesculitannin A [4], and its new epicatechin-(4α,8)-stereoisomeric analogue, respectively.

Owing to similar signal complexity of the ^{1}H NMR spectra as indicated for **5** and **7**, the structure of the remaining proanthocyanidin (**9**) was deduced from spectral analysis and chemical degradation. The absence of signals characterized by large coupling constants in the heterocyclic region suggested the constituent flavanyl units to possess the 2,3-*cis* configuration exclusively. This found support in the cleavage products, epicatechin and epicatechin-(4β,6)-epicatechin (**13**), generated by partial acid-catalysed hydrolysis and identified by TLC and HPLC comparison with authentic material. As the dimeric cleavage product **13**, preserving the mode of linkage between the DEF and GHI flavanyl units, originates from the chain terminating site, attachment of the epicatechin extension unit to dimer **13** required attention. Chromatographically, **9** clearly differed from authentic epicatechin-(4β,8)-epicatechin-(4β,8)-epicatechin in TLC mobility and in the R_ts on RP material. Evidence regarding the (4,6)-coupling of the 'upper' interflavanoid linkage in **9** was obtained from the downfield position of the aromatic A-

ring proton resonances (δ6.6–6.7) of its acetate (**10**) [7]. A strong positive Cotton effect in the 220–240 nm region of the CD spectrum of **9** indicated a 4β-flavanyl substituent. When the spectral data were considered in conjunction with the degradation products, the structure of **9** proved to be epicatechin-(4β,6)-epicatechin-(4β,6)-epicatechin, recently isolated from the same plant source [4] and *Kandelia candel* [14]. Its characterization, however, was primarily based on degradative techniques.

With reference to HPLC peak identification based on the CRD/UV absorption ratio (640/280 nm) [1], the proposed location of 'upper' (**1** and **3**) and 'lower' doubly linked units (**5** and **7**) as judged from their ratios 1.9 and 1.2, respectively, are in agreement with their established structures, as are the predominating epicatechin constituent units and (4,8)-interflavanyl linkages. Furthermore, a (4,6)-coupling in **9**, as confirmed by spectral data and degradation, initially followed from the enhanced absorption ratio 14.3, when compared to (4,8)-analogues displaying ratios within the range 7.0–11.0. Clearly, this approach provides tentative structural information, devoid of configurational conclusions, but, nevertheless, serves for prompt recognition of attractive polyphenolic metabolites for further structural elucidation.

EXPERIMENTAL

NMR spectra were recorded in acetone-d_6 and MeOH-d_4 (phenols) and $CDCl_3$ (acetates) with TMS as int. standard; CD data were obtained in MeOH. The FAB-MS were recorded with *m*-nitrobenzyl alcohol as matrix. For HPLC see ref. [1]. Acetylations were performed in Ac_2O–pyridine at room temp. Analyt. TLC was carried out on sheets [Kieselgel 60 F_{254}, 0.2 mm, Merck] using toluene–Me_2CO–HCO_2H (3:6:1) [system K1] as mobile phase.

Conversion of procyanidins into anthocyanidins. Procyanidin samples (2 mg) were dissolved in *n*-BuOH–HCl (2:1) and refluxed for 10 min. The anthocyanidins formed were identified by TLC on cellulose using HCl–*n*-BuOH–H_2O (3:30:10) as developing system.

Partial acid-catalysed degradation. Treatment of the procyanidin sample (*ca* 1 mg) with 0.5 M HCl according to ref. [15] furnished hydrolysates which were analysed by HPLC as recently described [1].

Extraction, isolation and identification of compounds. The plant material (750 g), fruit shells of *A. hippocastanum* L., collected at Freising, was exhaustively extracted with MeOH and the combined extracts evapd *in vacuo* to 800 ml, diluted with H_2O (3200 ml) and subsequently extracted with EtOAc. The organic phase gave on evapn of solvent, a brown solid (15 g), which was subjected to CC on Sephadex LH-20 (50 × 3.5 cm) with EtOH (3.6 l) as eluant. Frs (15 ml) were collected and combined according to their TLC behaviour. Tubes 25–75 contained (−)-epicatechin, B2 (R_f 0.62, system K1; DMACA: yellow-green; R_t 41.2 min; ratio $CRD_{640}UV_{280}$ 10.9), B5 (R_f 0.67, system K1; DMACA: green-blue; R_t 112.0 min; ratio $CRD_{640}UV_{280}$ 14.5), C1 (R_f 0.47, system K1; DMACA: green; R_t 63.6 min; ratio $CRD_{640}UV_{280}$ 7.7), and A2 (R_t 0.70, system K1; DMACA: blue; R_t 100.5 min; ratio $CRD_{640}UV_{280}$ 2.9).

Frs 85–99 (725 mg) were rechromatographed as described above (EtOH, 2.7°l). Following qualitative TLC analysis on cellulose [10% HCO_2H as solvent system; spray reagent DMACA: 1% dimethylaminocinnamaldehyde in EtOH and 6 M HCl (1:1)] appropriate frs (15 ml) were combined resulting in 8 major subfrs (A–H), which were lyophilized after removal of the organic phase. Final sepn was accomplished by prep. HPLC on a Polygosil 5C18 (30 × 2 cm) column, using a linear gradient from 5% HOAc–MeOH at a flow rate of 10 ml min^{-1}. From subfrs A and B: A2 (32 mg); B and C: B5 (17 mg); B, C and D: C1 (43 mg); E: **9** (35 mg); F and G: **7** (27 mg); F and G: **1** (89 mg); G and H: **3** (23 mg) and **5** (17 mg).

Epicatechin-(4β,8; 2β,7)*-epicatechin-*(4α,8)-ent-*epicatechin* (**1**). R_f 0.45 (system K 1; DMACA: blue). R_t 49.5 min; ratio CRD_{640}/UV_{280} 1.9. FAB-MS *m/z* 865 $[M + H]^+$, 713, 575, 533, 369, 337, 287. ^1H NMR (300 MHz, acetone-d_6): δ2.86 [*m*, H_2-4(I)], 3.41 [*d*, *J* = 3.5 Hz, H-3(C)], 4.13 [*d*, *J* = 3.5 Hz, H-4(C)], 3.90–5.34 [*m*, 5 × heterocyclic H], 5.74–6.18 [*m*, 4 × aromatic H (A, D, G)], 6.75–7.46 [*m*, 9 × aromatic H (B, E, H)]. CD: ref. [5].

Acetate (**2**). Acetylation and subsequent purification by prep. TLC (toluene–Me_2CO, 7:3) gave the acetate **2**. ^1H NMR: ref. [6].

Epicatechin-(4β,8; 2β,7)*-catechin-*(4β,8)*-epicatechin* (**3**). R_f 0.47 (system K 1; DMACA: blue). R_t 91.6 min; ratio CRD_{640}/UV_{280} 1.2. FAB-MS *m/z* 865 $[M + H]^+$. ^1H NMR (300 MHz, acetone-d_6): δ2.86 [*m*, H_2-4(I)], 3.45 [*d*, *J* = 3.5 Hz, H-3(C)], 3.97 [*d*, *J* = 3.5 Hz, H-4(C)], 3.51–4.80 [*m*, 5 × heterocyclic H], 5.80–6.18 [*m*, 4 × aromatic H (A, D, G)], 6.60–7.23 [*m*, 9 × aromatic H (B, E, H)].

Acetate (**4**). Acetylation and subsequent purification by prep. TLC (toluene–Me_2CO, 7:3) gave the acetate **4**. ^1H NMR (300 MHz, $CDCl_3$): δ1.60–2.40 (*m*, OAc), 2.90 [*m*, H_2-4(I)], 4.59 [*d*, *J* = 10.0 Hz, H-2(F)], 4.62 [*d*, *J* = 3.5 Hz, H-4(C)], 4.88 [*br s*, H-2(I)], 4.94 [*d*, *J* = 6.0 Hz, H-4(F)], 5.08 [*d*, *J* = 3.5 Hz, H-3(C)], 5.37 [*m*, H-3(I)], 5.84 [*m*, H-3(F)], 6.23 [*s*, H-6(G)], 6.41 [*d*, *J* = 2.2 Hz, H-6(A)], 6.61 [*s*, H-6(D)], 6.71 [*d*, *J* = 2.2 Hz, H-8(A)], 7.20–7.60 [*m*, 9 × aromatic H (B, E, H)]. CD $[\Theta]_{287}$ − 5175, $[\Theta]_{281}$ 0, $[\Theta]_{270}$ + 17300 $[\Theta]_{250}$ + 1700, $[\Theta]_{228}$ + 63170, $[\Theta]_{215}$ 0.

Epicatechin-(4β,8)*-epicatechin-*(4β,8; 2β,8)*-epicatechin* (**5**). R_f 0.59 (system K1; DMACA: green-grey). R_t 104 min; ratio CRD_{640}/UV_{280} 5.8. FAB-MS *m/z* 865 $[M + H]^+$. ^1H NMR (300 HMz, MeOH-d_4): δ2.80 [*m*, H_2-4(I)], 3.80–5.00 [*m*, 7 × heterocyclic H (C, F, I)], 5.92–6.12 [*m*, 4 × aromatic H (A, D, G)], 6.58–7.28 [*m*, 9 × aromatic H (B, E, H)].

Acetate (**6**). Acetylation and subsequent purification by prep. TLC (toluene–Me_2CO, 7:3) gave the acetate **7**. ^1H NMR (300 MHz, $CDCl_3$): δ1.6–2.4 (*m*, OAc), 2.90 [*m*, H_2-4(I)], 4.0–5.5 [*m*, 7 × heterocyclic H (C, F, I)], 6.45–6.80 [*m*, 4 × aromatic H (A, D, G)], 7.1–7.6 [*m*, 9

× aromatic H (B, E, H)]. CD $[\Theta]_{280}$ 0, $[\Theta]_{270}$ + 8300, $[\Theta]_{254}$ + 2900, $[\Theta]_{215}$ + 35 300.

Epicatechin-(4α,8)-epicatechin-(4β,8; 2β,7)-epicatechin (**7**). R_f 0.52 (system K1; DMACA: green-grey). R_t 82 min; ratio CRD_{640}/UV_{280} 4.6. FAB-MS *m/z* 865 $[M + H]^+$. ^1H NMR (300 MHz, acetone-d_6): δ2.9–5.1 [*m*, 9 × hetero cyclic H (C, F, I)], 6.0–6.2 [*m*, 4 × aromatic H (A, D, G)], 6.7–7.5 [*m*, 9 × aromatic H (B, E, I)].

Acetate (**8**). Acetylation and subsequent purification by prep. TLC (toluene–Me_2CO, 7:3) gave the acetate **8**. ^1H NMR (300 MHz, $CDCl_3$): δ1.6–2.4 (*m*, OAc), 2.80 [*m*, H_2-4(I)], 4.1–5.4 [*m*, 7 × heterocyclic H (C, F, I)], 6.2–6.5 [*m*, 4 × aromatic H (A, D, G)], 6.6–7.4 [*m*, 9 × aromatic H (B, E, H,)], CD $[\Theta]_{280}$ 0, $[\Theta]_{270}$ + 8400, $[\Theta]_{255}$ + 2900, $[\Theta]_{242}$ + 15 240, $[\Theta]_{225}$ − 34 265, $[\Theta]_{208}$ 0.

Reaction of **5** *and* **7** *with phloroglucinol.* Procyanidin samples (40 μg in 50 μl H_2O) and phloroglucinol (80 mg in 0.5 ml MeOH and 1.5 ml 0.5 N HCl; 100 μl) were refluxed at 100° for 5 min. The reaction mixt. was subjected to HPLC analysis [16] and the components identified by comparison with authentic material obtained from hydrolysis of known procyanidins.

Epicatechin-(4β,6)-epicatechin-(4β,6)-epicatechin (**9**). R_f 0.50 (system K1; DMACA: yellowish-green). R_t 126.9 min CRD_{640}/UV_{280} 14.3. FAB-MS *m/z* 889 $[M + Na]^+$. ^1H NMR (300 MHz, acetone-d_6): δ2.9–5.0 [*m*, 10 × heterocyclic H (C, F, I)], 6.0–6.2 [*m*, 4 × aromatic H (A, D, G)], 6.6–7.2 [*m*, 9 × aromatic H (B, E, H)].

Acetate (**10**). Acetylation and subsequent purification by prep. TLC (toulene–Me_2CO, 7:3) gave the acetate **10**. ^1H NMR (300 MHz, $CDCl_3$): δ1.6–2.4 (*m*, OAc) 2.90 [*m*, H_2-4(I)], 4.2–5.5 [*m*, 8 × heterocyclic H (C, F, I)], 6.6–6.7 [*m*, 4 × aromatic H (A, D, G)] 7.0–7.5 [*m*, 9 × aromatic H (B, E, H)]. CD $[\Theta]_{280}$ 0, $[\Theta]_{272}$ + 2500, $[\Theta]_{256}$ + 1220, $[\Theta]_{230}$ + 18630, $[\Theta]_{210}$ 0.

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