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Procyanidins from the herb of *Hypericum perforatum*

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From the aqueous acetone extract of the herb of *Hypericum perforatum* the flavanols catechin (**1**) and epicatechin (**2**), and the procyanidins A2 (**9**), B1 (**3**), B2 (**4**), B3 (**5**), B5 (**6**), B7 (**7**) and C1 (**8**) were isolated. Their structures were established as their peracetate derivatives, on the basis of chemical and spectral evidence. The ^{13}C NMR spectrum of the higher molecular weight polymer fraction revealed a 3′,4′-dihydroxylated B-ring oxidation pattern and the 2,3-*cis* relative stereochemistry of the constituent flavan-3-ol units. The mean average molecular size of the polymers was estimated to be 4 to 5 flavan-3-ol units. The procyanidin pattern in comparison to that of *Crataegus* spec. is briefly discussed.

1. Introduction

Extracts of *Hypericum perforatum* L. (St. John's wort, Clusiaceae) are widely used in phytomedicine for the treatment of mild and moderate depression [1]. The tannin fraction of the herb of *H. perforatum* was recently recognized to consist of oligomeric procyanidins; previous investigations on the tannin fraction demonstrated the presence of procyanidin B2 and has elaborated hints on the occurrence of an additional dimeric, two trimeric and a tetrameric procyanidin [2]. The pattern of the tannin fraction was expected to be closely related to that of *Crataegus* (hawthorn) species with similar vasoactive properties [2, 3]. Furthermore, a recent study reports on the increase of the solubility of the naphthodianthrones hypericin and pseudohypericin in the presence of a fraction containing procyanidins as well as the isolated procyanidins B2 and C1; pure hypericins thus solubilized produced a remarkable decrease of the duration of immobility in the forced swimming test [4] which is indicative of antidepressant activity. In the present study the proanthocyanidins of *H. perforatum* are therefore investigated in more detail.

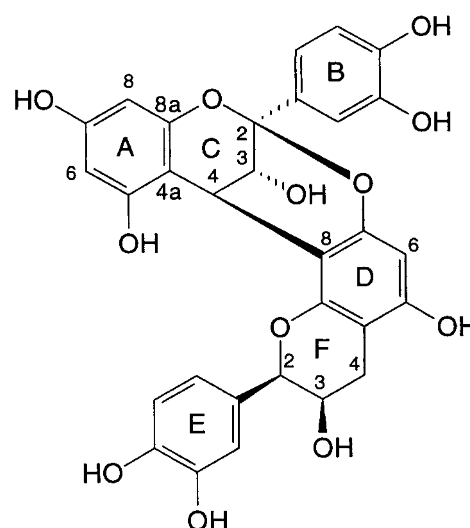
2. Investigations, results and discussion

The ethylacetate fraction obtained from the aqueous acetone extract of the herb of *Hypericum perforatum* (Hyperici herba) was chromatographed on Sephadex LH-20. Fractions containing oligoflavanoids were further purified by HSCCC, MCI gel chromatography and MPLC on RP-18 material to give compounds **1**–**9**. The identity of all flavanoids was established by physical properties (1D- and 2D NMR, circular dicroism (CD), [α]ₗ and MALDI-TOF-MS) of the corresponding derivatives obtained after peracetylation.

Compounds **1** and **2** were readily identified by the spectroscopic data of their peracetates as the monomeric flavanols catechin and epicatechin in comparison with authentic material; both compounds were first detected in *H. perforatum* in 1974 [5]. Compounds **3**–**7** were identified as the dimeric procyanidins B1 (epicatechin-(4β→8)-catechin; **3**), B2 (epicatechin-(4β→8)-epicatechin; **4**), B3 (catechin-(4α→8)-catechin; **5**), B5 (epicatechin-(4β→6)-epicatechin; **6**) and B7 (epicatechin-(4β→6)-catechin; **7**) through spectroscopic data of their peracetates, which were identical with those previously described [6–8]. ^{1}H NMR data of the peracetate derivative of the trimeric procyanidin C1 (epicatechin-(4β→8)-epicatechin-(4β→8)-epicatechin; **8**) are in line with those previously described [9, 10] and agree with the assumption of Melzer [2], who chromatographically compared an oligomeric procyanidin fraction of *Hypericum* extract with that of *Crataegus* extract. Compound **9** was identified as procyanidin A2 (epicatechin-(4β→8, 2β→O→7)-epicatechin). The NMR data set of its peracetate (s. Exp.) agreed to a large extent with published data [11]. Compounds **3** and **5** to **9** are unambigiously described here for the first time as constituents of the crude drug material of *H. perforatum*.

The structural resemblance of the lower molecular weight flavanols **1**–**9** to the procyanidin pattern of *Crataegus* species [12] is obvious. Although type-A procyanidins were hitherto not detected in European *Crataegus* species, the occurrence of procyanidin A2 in *C. sinaica* was recently demonstrated [13] and suggests that this class of procyanidins is probably more widespread in the genus *Crataegus*.

However, little is known about the structural design of the polymeric procyanidin fraction of the title plant. This knowledge is of importance for a better understanding of the chemical structure of the proanthocyanidins in relation to their influence on the solubility of compounds with antidepressant activity in phytomedicines produced from *H. perforatum* [4]. In addition, the observed vasoactive properties of *H. perforatum* extract [3] may be connected with procyanidins with higher degree of polymerization as observed for procyanidins of *Crataegus* extracts [14].



Procyanidin A 2

The polymer fraction (obtained and defined as described elsewhere [18]) showed an optical rotation of $[\alpha]^{20}_{578}$ +77° (*c*0.1; MeOH) which corresponds to a molar proportion of subunits with 2,3-*cis* stereochemistry of 83% [15]. This result was further confirmed by ^{13}C NMR spectroscopy of the polymer fraction. The ^{13}C NMR spectrum (Fig.) revealed that the flavan monomers not only exhibit a 3′,4′-dihydroxylated B-ring oxidation pattern (114–118 ppm) but also show 2,3-*cis* relative stereochemistry [16]. Unfortunately, the relative *cis/trans* proportion cannot be calculated from the ^{13}C NMR spectrum by the C-2 signal intensities of constituent extender units at 77 ppm (2,3-*cis*) and 84 ppm (2,3-*trans*), because the later signal is too weak for accurate measurement. The mean average molecular size of the polymers was estimated for 4 to 5 flavan-3-ol units by integration of the C-3 signals of the extender units at 73 ppm and the corresponding signal of the lower flavan-3-ols at 68 ppm [17].

When the polymer fraction was degraded in the presence of phloroglucinol under acidic conditions at ambient temperature for 30 min the degradation products were epicatechin, epicatechin-(4β→2)-phloroglucinol, procyanidin B2 and epicatechin-(4β→8)-epicatechin-(4β→2)-phloroglucinol; this suggests that the polymeric fraction was principally built from epicatechin as terminating flavan-3-ol unit and epicatechin as extender units with predominantly 4→8 interflavanoid linkages.

The determination of general structural features of the *Crataegus* polymeric fraction (obtained in the same way as the *Hypericum* polymeric fraction) by ^{13}C NMR demonstrated close structural resemblance of both preparations. The ^{13}C NMR spectrum showed exclusively the 3′,4′-dihydroxylation and the dominance of 2,3-*cis* configurated constituent flavan-3-ol units as well (s. Fig.). The optical rotation of $[\alpha]^{20}_{578}$ +109° (*c*0.1; MeOH) corresponds to a molar proportion of subunits with a relative 2,3-*cis* stereochemistry of 89% [15]. Only the mean average molecular size with 6 to 7 flavan-3-ol units differs slightly from that of the *Hypericum* polymeric fraction.

These results indicate a great similarity in the chemical composition of the procyanidins from *Crataegus* spec. and those of *Hypericum perforatum.* Thus, the vasoactive properties observed with *H. perforatum* [2, 3] are most probably due to the analogous procyanidins based on 4→8 linked epicatechin moieties.

3. Experimental

3.1. General procedures

^{1}H NMR spectra were recorded in $CDCl_3$ (except for the polymers, see Fig.) on a Varian Gemini 200 (200 MHz) or a Bruker AM 600 (600 MHz), relative to $CHCl_3$. ^{13}C NMR were recorded at 50 or 150 MHz, CD spectra in MeOH on a Jasco J 600. Acetylation was performed in Ac_2O-pyridine (1,2 : 1) at ambient temperature for 24 h. MALDI-TOF mass spectrometer: LAZARUS II (home built), N2-laser (LSI VSL337ND) 337 nm, 3 ns puls width, focus diameter 0.1 mm, 16 kV acceleration voltage, 1 m drift length, data logging with LeCroy9450A, 2.5 ns sampling time and expected mass accuracy ±0.1%; sample preparation: acetylated compounds were deposited from a solution in $CHCl_3$ on a thin layer of 2,5-dihydroxybenzoic acid (DHB) crystals. Analytical TLC was done on silica gel GF_{254} plates (Merck) in the solvent system EtOAc-HCO_2H-H_2O (18 : 1 : 1). Compounds were visualized as red spots by spraying with vanillin/HCl-reagent. Preperative TLC was performed on silica gel plates (Kieselgel 60 F_{254}, 0.5 mm, Merck) using EtOAc–HCO_2H–H_2O (18 : 1:1) for free phenols and toluene-Me_2CO (7 : 3) for peracetate derivatives. Optical rotation ([α]) was measured using a Perkin-Elmer polarimeter 241. High speed countercurrent chromatography (HSCCC) was carried out with the solvent system $CHCl_3$–MeOH–H_2O 65 : 35 : 20 on P.C. Inc. Separator-Extractor, 325 ml coil, flow rate 1.0 ml^{-1} at 800 rpm, using the upper-phase as mobile phase.

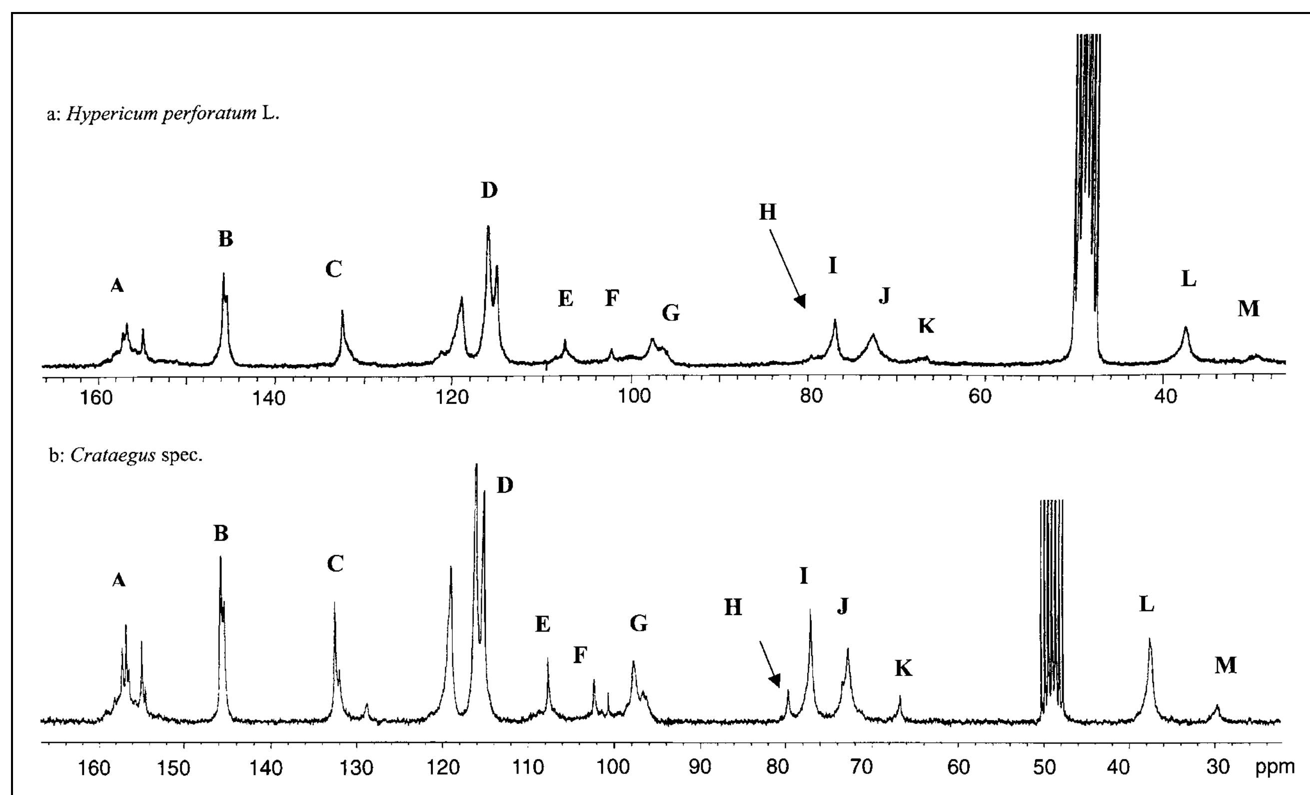


Fig. 1: Determination of general structural features of *Hypericum perforatum* polymeric fraction (a) and of *Crataegus* spec. polymeric fraction (b) by 50,29 MHz ^{13}C NMR (solvent: methanol-*d*$_4$). (A) C-5, C-8a and C-7 of PC units; (B) C-3′ und C-4′ of PC units; (C) C-1′ of PC units; (D) C-2′, C-5′ und C-6′ of PC units; (E) substituted A-ring carbons; (F) C-4a of PC units: (G) C-6 and C-8 of PC units; (H) C-2 of the terminal PC units; (I) C-2 from *2,3-cis* PC units; (J) C-3 of all PC units; (K) C-3 of the terminal flavan-3-ol units; (L) C-4 of extender flavanol units; (M) C-4 of terminating flavanol units

3.2. Plant material

The herb of *Hypericum perforatum* (Herba Hyperici conc.; DAC 1986, 3 Ergb. 91; Ch.: 60320306) was obtained from Caelo, Hilden, Germany. A voucher specimen (PBMS 141) is deposited at the Herbarium of the Institute. Crataegi folium cum flore was purchased from Müggenburg, Hamburg, Germany (Ch.: 48160; voucher specimen PBMS 142).

3.3. Extraction and isolation

The herb of *Hypericum perforatum* (1 kg) was extracted with acetone−H_2O 7 : 3 (7.5 l) at room temperature. The water fraction was concentrated, filtered to remove chlorophyll and defatted with petrol (20 × 200 ml). Extraction of the defatted residue with ethylacetate (100 × 200 ml) gave a brown solid (31 g). The ethylacetate fraction (25 g) was subjected to CC on Sephadex LH-20 (5.5 × 68 cm) with EtOH−H_2O 3 : 1 (11 l), EtOH−MeOH 1 : 1 (8 l), MeOH (4 l) and acetone−H_2O 7 : 3 (3 l) to give 12 fractions. Fraction 4 (4880−6300 ml, 0.96 g) was applied to MPLC (Orpegen RP-18, 18−30−60 μm, 26 × 460 mm, Büchi) using a 20−50% MeOH linear gradient; 2 l) to give compound **1** (1680 ml; 500 mg) and **2** (1681−1700 ml; 16 mg). Sephadex fraction 5 (6331−7420 ml, 1,2 g) was purified by HSCCC (see above) to give subfraction 5a (22 ml, 62 mg), followed by MCI gel CHP-20 P (2,5 × 25 cm, 75−100 μm; Mitsubishi Kasei Corporation, Tokyo) chromatography using a 10−80% MeOH (2 l) linear gradient to give a mixture of compound **3** and **5** (1088 ml, 17 mg), compound **4** (1089−1448 ml, 10 mg) and **8** (1449−1568 ml; 6 mg). Final purification of compounds **3** and **5** was achieved on preparative TLC after acetylation of the mixture. Sephadex fraction 6 (7421−8680 ml, 0,45 g) was separated by HSCCC (see above) to give subfraction 6a (44 ml, 126 mg), followed by chromatography on the same MCI gel system as above (1184 ml, 26 mg). Final purification was done on preparative TLC to yield compound **6** (14 mg) and **9** (8 mg). Compound **7** (10 mg) was obtained from the Sephadex fraction 7 (8681−10200 ml, 0,54 g), followed by HSCCC (21 ml, 380 mg) and MCI gel chromatography (728 ml, 88 mg) described as above and final purification on preparative TLC. Compound **9** (procyanidin A2); peracetate derivative: MALDI-TOF-MS: 977 $[M + Na]^+$; $[\alpha]_D^{20}$ −50° (*c*0,1, acetone); ^1H NMR ($CDCl_3$, 600 MHz, δ) = 1.1−2.4 (m, OAc), 2.82 [dd, J = 2.9 and 17.2 Hz, H-4eq (F)], 2.92 [dd, J = 4.7 and 17.2, H-4ax (F)], 5.21 [brs, H-2 (F)], 5.27 [m, H-3 (F)], 4.60 [d, J = 4 Hz, H-4 (C)], 5.19 [d, J = 4 Hz, H-3 (C)], 6.50 [s, H-6 (D)], 6.49 [d, J = 2.2 Hz, H-6 (A)], 6.81 [d, J = 2.2 Hz, H-8 (A)], 7.20 [d, J = 8.4 Hz, H-5 (E)], 7.28 [dd, J = 2.2 and 8.4 Hz, H-6 (E)], 7.33 [d, J = 2.2 Hz, H-2 (E)], 7.25 [d, J = 8.4 Hz, H-5 (B)], 7.56 [dd, J = 2.2 and 8.4 Hz, H-6 (B)], 7.47 [d, J = 2.2 Hz, H-2 (B)]; ^{13}C NMR ($CDCl_3$, 150 MHz) = 25.2 [C-4 (F)], 27.3 [C-4 (C)], 66.2 [C-3 (F)], 67.2 [C-3 (C)], 77.6 [C-2 (F)], 97.7 [C-2 (C)], 103.9 [C-6 (D)], 105.5 [C-4a (D)], 107.1 [C-8 (A)], 108.8 [C-4a (A)], 109.7 [C-6 (A)], 113.4 [C-8 (D)], 122.9 [C-2 (B)], 123.0 [C-5 (B)], 123.1 [C-5 (E)], 124.1 [C-2 (E)], 125.1 [C-6 (B)], 125.8 [C-6 (E)], 135.1 [C-1 (E)], 135.4 [C-1 (B)], 141.5 [C-3 (B)], 141.9 [C-3 (E)], 142.6 [C-4 (E)], 142.9 [C-4 (B)], 148.2 [C-5 (A)], 148.8 [C-5 (D)], 149.9 [C-7 (A)], 150.1 [C-7 (D)], 151.9 [C-8a (D)], 153.8 [C-8a (A)]. CD: $[H]_{284}$ −11413, $[\Theta]_{270}$ +31525, $[\Theta]_{238}$ −8079.

3.4. Preparation of polymeric fractions

Preparation of the polymeric fractions was achieved and defined according the procedure described by Koupai-Abyazani and Bohm [18].

The water fraction obtained after extraction of the drug material (46 g) was fractionated by CC on Sephadex LH-20 (5,5 × 68 cm) with MeOH−H_2O 1 : 1 (18 l) until the eluant was colourless; then acetone-H_2O 7 : 3 (4 l) was used for elution. The acetone-H_2O-eluant (9 g) was further fractionated on Sephadex LH-20 (3 × 29 cm) with dioxane-H_2O 7 : 3 (2 l) to remove hypericins. The polymeric fraction (2,3 g) was obtained after 500 ml.

Crataegi folium cum flore (500 g) was extracted with acetone−H_2O 7 : 3 (5,6 l), reduced in volume and defatted with petrol (5 × 200 ml). The concentrated aqueous phase was extracted with EtOAc (6 l) to give a residue of 7,6 g. The remaining H_2O-phase (30 g) was subjected to CC on Sephadex LH-20 (see above) using MeOH-H_20 1 : 1 (6 l) and acetone-H_2O 7 : 3 as eluants to give 4,9 g of the polymeric fraction.

3.5. Degradation with phloroglucinol

The polymeric fraction of *Hypericum* obtained as described above (800 mg) was treated for 30 min at room temperature with phloroglucinol (530 mg) in 1% HCl in EtOH (30 ml) under continuous shaking [19]. The solution was concentrated under reduced pressure (1320 mg). A portion (870 mg) was fractionated by CC on Sephadex LH-20 (5,5 × 68 cm) using EtOH 96% (3,5 l), EtOH-MeOH 1 : 1 (3 l), acetone-H_2O 7 : 3 (2 l) as eluants to give 8 fractions. Fraction 2 (1727 ml, 15 mg) was purified by MCI gel chromatography (see above) using a 10−80% MeOH linear gradient (2 l) yielding epicatechin (1105 ml, 10 mg). Fraction 4 (2882−3718 ml, 140 mg) was chromatographed on MCI gel to yield epicatechin-(4β→2)-phloroglucinol (1232 ml, 27 mg) and procyanidin B2 (5 mg). Epicatechin-(4β→8)-epicatechin-(4β→2)-phloroglucinol (37 mg) was isolated from Sephadex fraction 7 (4235−4642 ml, 55 mg) followed by MCI gel chromatography (816 ml) under the conditions described.

The structures of the monomeric flavan-3-ols and the phloroglucinol derivatives were determined on the basis of their 1D- and 2D-NMR (HSQC, HMBC), CD and $[\alpha]_D^{20}$ data of their peracetylated derivatives.

Epicatechin-(4β→2)-phloroglucinol-peracetate: MALDI-TOF-MS: 773 $[M + Na]^+$; $[\alpha]_D^{20}$ +62.5° (*c*0.112, MeOH); ^1H NMR ($CDCl_3$, 600 MHz, δ): 1.84−2.33 (m, OAc), 4.45 [d, J = 2.3 Hz, H-4 (C)], 5.12 [dd, J = 2.3 and <1 Hz, H-3 (C)], 5.46 [brs, H-2 (C)], 6.60 [d, J = 2.3 Hz, H-6 (A)], 6.74 [d, J = 2.3 Hz, H-8 (A)], 6.83 [d, J = 2.3 Hz, H-4 or H-6 (D)], 6.97 [d, J = 2.3 Hz, H-6 or H-4 (D)], 7.18 [d, J = 8.4, H-5 (B)], 7.22 [dd, J = 8.4 and 1.8, H-6 (B)], 7.32 [d, J = 1.8 Hz, H-2 (B)]. ^{13}C NMR ($CDCl_3$, 150 MHz): 33.9 [C-4 (C)], 70.8 [C-3 (C)], 73.9 [C-2 (C)], 107.7 [C-8 (A)], 108.8 [C-6 (A)], 110.4 [C-4a (A)], 114.4 [C-4 (D)], 115.4 [C-6 (D)], 120.7 [C-2 (D)], 122.2 [C-2 (B)], 123.2 [C-5 (B)], 124.6 [C-6 (B)], 135.8 [C-1 (B)], 141.9 [C-3 (B)], 142.0 [C-4 (B)], 149.7−150.2 [C-7 (A), C-5 (A), C-1 (D), C-3 (D), C-5 (D)], 154.9 [C-8a (A)]. CD: $[\Theta]_{279}$ +6597, $[\Theta]_{235}$ +43231.

Epicatechin-(4β→8)-epicatechin-(4β→2)-phloroglucinol-peracetate: MALDI-TOF-MS: 1272 $[M + Na]^+$; $[\alpha]_D^{20}$ +90° (*c*0.1, MeOH); ^1H NMR ($CDCl_3$, 600 MHz, δ, r = rotameric form): 1.2−2.4 (m, OAc), 4.39 [brs, H-4 (F)], 4.43 [brs, Hr-4 (F)], 4.48 [d, H-4 (C)], 4.71 [brs, Hr-4 (C)], 4.85 [brs, H-2 (F)], 4.92 [m, H-3 (C)], 5.04 [m, H-3 (F)], 5.29 [brs, Hr-2 (C)], 5.33 [m, Hr-3 (F)], 5.45 [m, Hr-3 (C)], 5.49 [brs, Hr-2 (F)], 5.69 [brs, H-2 (C)], 6.03, 6.26, 6.67, 6.74 [all d, J = 2.5 Hz, H-6 (A), Hr-6 (A), H-8 (A), Hr-8 (A)], 6.65 and 6.85 [s, H-6 (D) or Hr-6 (D)], 6.84, 6.86, 6.94, 7.03 [all d, J = 2.5 Hz, H-4 (G), Hr-4 (G), H-6 (G), Hr-6 (G)], 6.82−7.32 (2 × B- and E-ring protons); ^{13}C NMR ($CDCl_3$, 150 MHz): 32.8 [C-4 (C)], 34.7 [Cr-4 (C)], 35.2 [Cr-4 (F)], 35.8 [C-4 (F)], 70.0 [Cr-3 (C)], 70.9 [C-3 (C)], 71.3 [C-3 (F)], 71.4 [Cr-3 (F)], 73.5 [C-2 (C)], 74.3 [Cr-2 (F)], 74.7 [Cr-2 (C)], 75.4 [C-2 (F)], 107.4, 107.5, 108.0, 109.1 [C-6 (A) and C-8 (A) or Cr-6 (A) and Cr-8 (A)], 110.5, 110.9 [C-6 (D) and Cr-6 (D)], 110.93 [C-4a (D)], 111.3 [C-4a (A)], 114.5, 114.6, 114.9, 115.0 [C-4 (G), Cr-4 (G), C-6 (G), Cr-6 (G)], 120.5−125.8 [2 × B- and E-ring carbons], 151.9 [C-8a (A)], 152.0 [C-8a (D)]. CD: $[\Theta]_{280}$ +2604, $[\Theta]_{237}$ +6173.

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