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Polymeric Proanthocyanidins from *Psidium guajava*

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Abstract

From the aqueous acetone extract of the leaves of *Psidium guajava* L. the flavanols catechin and gallocatechin, the procyanidins B1, B2, B3 and two prodelphinidins (gallocatechin - (4α→8) - catechin; gallocatechin - (4α→8) - gallocatechin) were isolated. A more abundant proanthocyanidin polymer was also isolated, purified and its chemical constitution studied by ^{13}C-NMR and phloroglucinol degradation. The mean molecular weight of the polymer was estimated to be about 9 to 10 flavan-3-ol-units with a ratio of procyanidin : prodelphinidin units at 2 : 1 some of which are derivatized by gallic acid.

Keywords

Psidium guajava, Myrtaceae, proanthocyanidins, phloroglucinol degradation

Introduction

Psidium guajava L. (Myrtaceae) has been widely used in folk medicine. The fruits are edible and the juice is used as a refreshing drink [1]. In addition, it has been reported that the leaves have shown antimalarial, spasmolytic, antidiarrheal, antimicrobial, anti-inflammatory, analgesic, antipyretic, CNS depressant, hypoglycaemic activity, and anticough activities [2-8]. The leaves of *Psidium guajava* contain an essential oil, resin, sugars, triterpenes, flavonoids, ellagitannins, flavan-3-ols and proanthocyandins [9-16]. Among the flavan-3-ols and the

proanthocyanidins, catechin, galloactechin, procyanidin B-1, procyanidin B-2, procyanidin B-3 and epigallocatechin-(4β→8)-gallocatechin (prodelphinidin B-1) were isolated from the leaves or the bark of *Psidium guajava* [12,14,16]. Several pharmacological activities, including antiphlogistic, antioxidant, antifungal, antitumor effects, are reported for higher oligomeric and polymeric proanthocyanidins [17]. Little is known about the structural design of the polymeric proanthocyanidin fraction of the title plant. However, this knowledge is of importance for a better understanding of the chemical structure of the proanthocyanidins in relation to their pharmacological and microbiological effects.

Results and Discussion

The ethylacetate fraction obtained from the aqueous acetone extract of the leaves of *Psidium guajava* was chromatographed on Sephadex LH-20 and MCI gel chromatography (s.Exp.). Two dimeric prodelphinidins, gallocatechin-(4α→8)-catechin and gallocatechin-(4α→8)-gallocatechin have been isolated in addition to the known flavanols catechin (**1**) and gallocatechin (**2**) as well as the dimeric procyanidins epicatechin-(4β→8)-catechin (procyanidin B1, **7**), epicatechin-(4β→8)-epicatechin (procyanidin B2) and catechin-(4α→8)-catechin (procyanidin B3). The identity of all flavanoids was established by physical properties (1D-and 2D NMR, circular dicroism (CD), optical rotation [α], and MALDI-TOF-MS) of the corresponding derivatives obtained after peracetylation, and compared with authentic samples and published data [18, 19, 32].

The polymer fraction (obtained s.Exp., **fig.1**) showed an optical rotation of +123° (*c* 0.1; MeOH) corresponds to a molar proportion of subunits with a relative 2,3-*cis* stereochemistry of 92% [20]. The ratio of procyanidin : prodelphinidin (PC:PD) obtained by spectrophotometric measurement [21] of the hydrolysis products was estimated to be 67:33. Unfortunately, the relative *cis/trans* proportion could not be corroborated by ^{13}C-NMR spectroscopy (solvent: MeOH-d_4, 99 MHz) of the polymer fraction by the C-2 signal intensities of constituent extender unit at

δ 77 ppm (2,3-*cis*) and 84 ppm (2,3-*trans*) [22], because the latter signal was too weak for accurate measurement. The mean average molecular size of the polymers was estimated to be 9-10 flavan-3-ol units by integration of the corresponding signal of the lower flavan-3-ols at 68 ppm [23]. Integration of the signals at δ 115-116 ppm and 107 ppm led to the estimation of a 2:1 ratio of the procyanidin : prodelphinidin units [24,25] thus corroborating the ratio obtained by spectrophotometric measurement. The presence of gallate units was obvious by the carbon chemical shift at 110, 122 and 139 ppm as well as the carbonyl carbon chemical shift at δ 166 ppm [26].

To elucidate the structure in more detail, the polymer was subjected to mild acid catalysed scission, and the generated extender flavan carbocations were captured with phloroglucinol as described [27, 30].

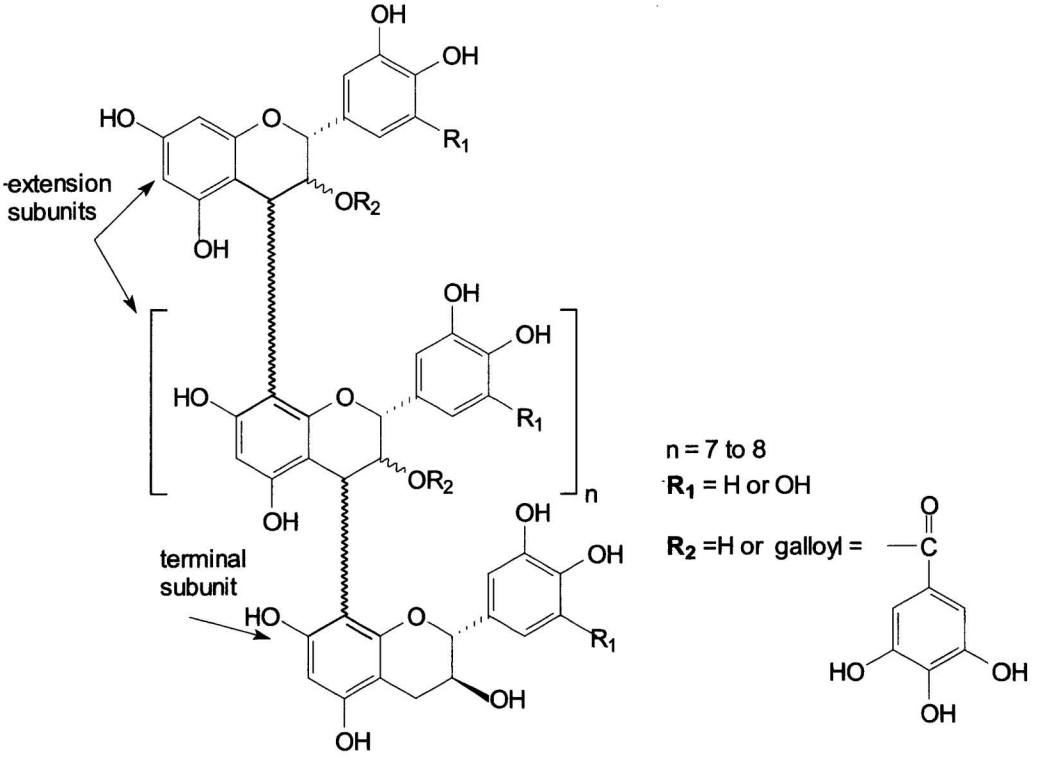

Fig. 1. General structure of polymeric proanthocyanidins from *Psidium guajava* leaves.

The reaction resulted in the cleavage of the terminal flavanoid units, which where identified as catechin (**1**) and gallocatechin (**2**). A small amount of the procyanidin dimer, epicatechin-(4β→8)-catechin (**7**) was also isolated from the reaction mixture, confirming the propensity of catechin as end unit. Among the monomeric phloroglucinol captured products epicatechin-(4β→2)-phloroglucinol (**3**), epigallocatechin-(4β→2)-phloroglucinol (**4**), epicatechin-3-O-gallat-(4β→2)-phloroglucinol (**5**) and epigallocatechin-3-O-gallat-(4β→2)-phloroglucinol (**6**) were isolated (**fig. 2**).

All compounds were identified by ^{1}H-NMR, MALDI-TOF-MS and circular dicroism (CD), and [α] of the corresponding derivatives obtained after peracetylation. Comparison of the data with previously published values [28-33], identified **1-7** as such.

To establish the type of the interflavanoid linkages as 4→8 or 4→6 in the polymer two larger scission products with clearly defined linkages were isolated: Epicatechin-3 -O-gallat- (4β→8) -epicatechin- 3 -O -gallat- (4β→2)- phloroglucinol (**8**) and epicatechin-(4β→8)-catechin (**7**). This suggests the predominance of 4→8 interflavanoid linkages in the polymeric fraction. The structure of **8** has been elucidated by NMR spectrocopic data of its peracetate (**8a**). It showed a prominent pseudomolecular ion peak at m/z 1744 [M + Na$^+$] the MALDI-TOF-MS M + Na$^+$, that is indicative of a dimeric proanthocyanidin derivative composed of two catechin/epicatechin units, two galloyl moieties and one phloroglucinol ring. Its ^{1}H-NMR spectrum is very similar to that of the analogous dimeric phloroglucinol derivative epigallocatechin-3-O-gallat-(4 β→8)-epigallocatechin-3-O-gallat-(4β→2)-phloroglucinol-peracetate [32] except for two AMX-spin systems typical for catechol-type–B rings. The heterocyclic coupling constants ($J_{2,3}$ < 2 MH) confirmed the relative 2,3-*cis* configuration of ´upper` and ´lower` constituent units. A diagnostic feature in the ^{1}H-NMR spectrum was the presence of two sharp low-field two proton singlets (δ 7.33 and 7.43 ppm), attributable to the equivalent protons of two galloyl groups. The structure elucidation was corroborated by acid-catalysed

reaction of **8** in the presence of phloroglucinol to give epicatechin-3-O-gallat-(4β→2)-phloroglucinol (**5**) as major addition product. The high amplitude positive cotton effect at 200-240 nm in the CD spectrum of **8a** confirmed the absolute configuration as 4R [34, 35]. The best of our knowledge, compound **8** is here described for the first time. The NMR-data of the peracetate derivative **8a** were here reported for the first time.

In conclusion, 2 flavanols and five dimeric proanthocyanidins have been isolated and identified from an acetone-water extract of *Psidium guajava*. The ^{13}C NMR and the phloroglucinol degradation of the polymeric mixture showed the predominance of 2,3-*cis*-configurated flavane-3-ol units with mostly dihydroxylated B-rings. The presence of galloylated derivatives in the polymeric proanthocyanidin mixture and the absence of such derivatives in the low molecular polyphenol fractions posed the question to the biogenetically regulation of the galloylation step. Future investigations deal with the pharmacological and microbiological testing of individual compounds and the polymeric mixture are necessary [36].

Experimental

General

^1H NMR spectra were recorded in $CDCl_3$ (except for the polymeric fraction in MeOH-d_4) on a Varian Mercury 400 plus. CD spectra were measured in MeOH on a CD spectrometer AVIV 62A DS. Acetylation was performed in Ac_2O-pyridine (1,2:1) at ambient temperature for 24 h. MALDI-TOF mass spectrometer: LAZARUS II (home built), N_2-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 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) with the mobile phase EtOAc-HCO_2H-H_2O (18:1:1). Compounds were visualized as red spots by spraying with vanillin/HCl-reagent. Preparative TLC

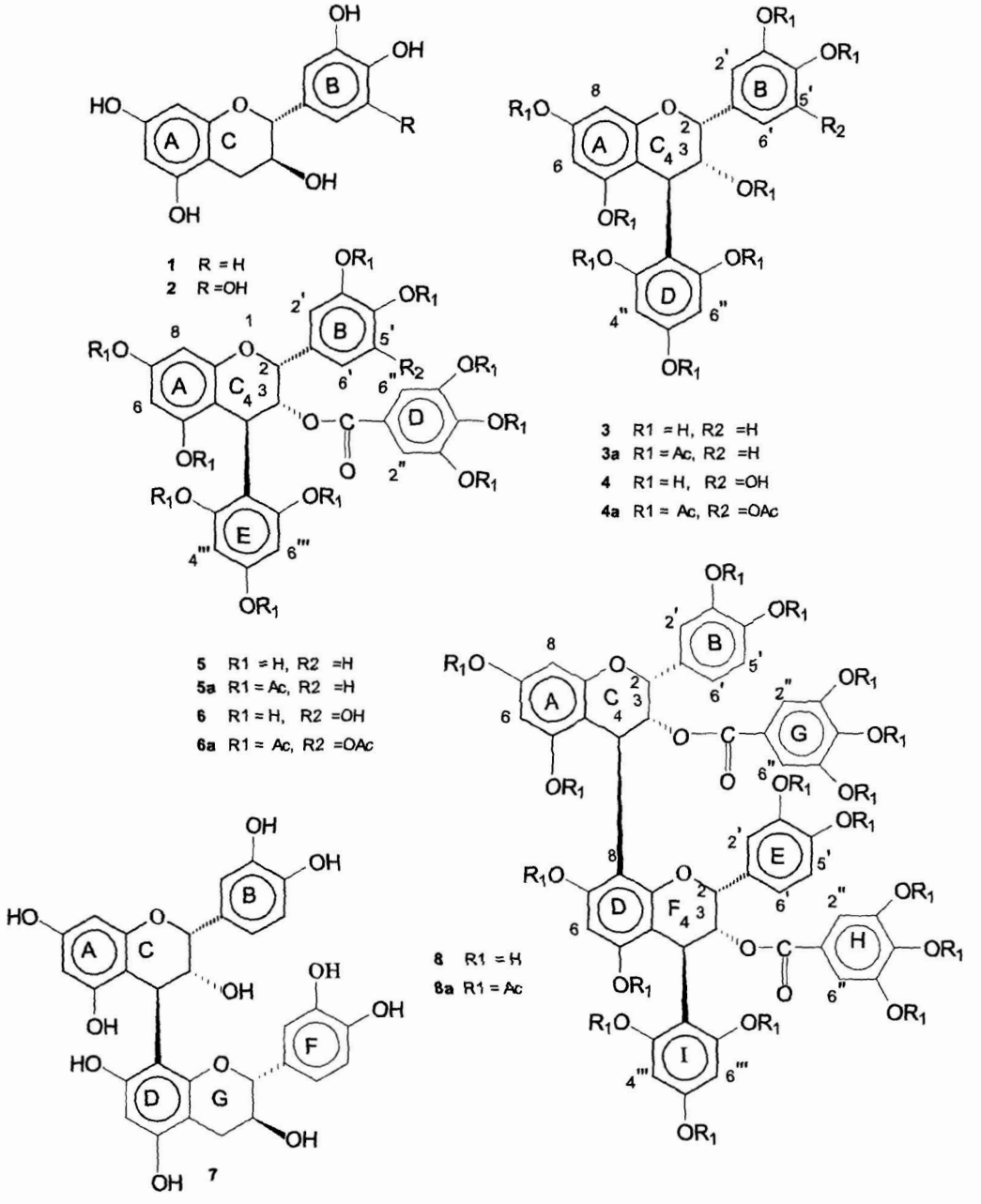

Fig. 2. Structure of the subunit products found in *Psidium guajava* leaves following acid-catalysis in the presence of phloroglucinol.

was performed on silica gel plates (Kieselgel 60 F254, 0.5 mm, Merck) using toluene-Me_2CO (7: 3) for peracetates. Optical rotation ([α]) was measured using a Perkin-Elmer polarimeter 241.

Plant material

Psidium guajava L. leaves were collected from trees grown in Sudan (07/2001) and identified in comparison with authentic *Psidium guajava* obtained from the University of Jordan/Amman. A voucher specimen is deposited at the University of Petra (no. 0701).

Extraction and isolation

The air-dried material (2 kg) was exhaustively extracted with Me_2CO/H_2O (7 : 3, 12 l) and the combined extracts evaporated *in vacuo* to 1.5 l, filtered to remove the precipitated chlorophyll, concentrated and defatted with petrol. Successive extractions with EtOAc (7.5 l) gave, on evapn of solvent solid of 40.3g EtOAc fraction. The remaining H_2O-phase (WP) was evaporated to dryness (204 g). 30.0 of the EtOAc fraction were subjected to CC on Sephadex LH-20 (5.5x 68 cm) with EtOH-H_2O (6 l), EtOH-MeOH 1:1 (7 l), MeOH (3l) and acetone-H_2O 7:3 (4 l) to give 10 fractions. Fraction 3 (3800-4200 ml, 1.3 g) was subjected to chromatography on MCI-gel CHP 20 P (25 x 250 mm) with a 10-80 % MeOH linear gradient (17 ml/frs.) to afford catechin **1** (subfrs. 29-41, 223 mg) and gallocatechin **2** (subfrs. 69-89, 450 mg). Fraction 4 (4200-4770 ml, 2.2 g) was separated on MCI-gel with the same gradient as above to afford epicatechin-(4β→8)-epicatechin (procyanidin B-2; subfrs. 90-105, 270 mg). Fraction 6 (5600-6700 ml, 3.8 g) was separated on MCI-gel to afford subfrs. 87-115 (740 mg). A portion of the subfrs. were acetylated and purified on prep. TLC to yield the peracetates of epicatechin-(4β→8)-catechin (procyanidin B-1; 17 mg) and catechin-(4α→8)-catechin (procyanidin B-3; 9 mg). Gallocatechin-(4α→8)-catechin was achieved from fraction 8 (9200-9700 ml, 300 mg) followed by MCI-gel chromatography (subfrs. 41-49, 27 mg). Gallocatechin-(4α→8)-gallcatechin was isolated from fraction 9 (9700-990 ml, 150 mg) followed by MCI-gel chromatography as described above (subfrs. 26-31, 18 mg). All

compounds were identified after acetylation by their physical data (NMR, MS, CD) and by comparison with authentic samples and published values [18,19,32].

Preparation of polymeric fraction

Preparation of the polymeric fraction was achieved and defined according the procedure described by Foo et al. [27,30]. The water-phase (WP) obtained after extraction of the plant material (204 g) was fractionated by CC on Sephadex LH-20 (5.5x 68 cm) with MeOH-H_2O 1:1 (20 l) until the eluent was colourless; then acetone-H_2O 7:3 (7 l) was used for elution to obtain the polymeric fraction (19 g).

Degradation with phloroglucinol

The polymeric fraction of *Psidium guajava* obtained as described above (10 g) was treated for 30 min. at room temperature with phloroglucinol (6 g) in 1% HCl in EtOH (50 ml) under continuous shaking [27,30]. The solution was reduced with pressure (15.8 g). A portion (6 g) was fractionated on Sephadex LH-20 (5.5x 68 cm) using EtOH (5 l), EtOH-MeOH 1:1 (5 l) and acetone-H_2O 7:3 (3 l) to give 8 fractions. Frs.2 (3550-3900 ml, 430 mg) was subjected to chromatography on MCI-gel CHP 20 P (25 x 250 mm) with a 10-80 % MeOH linear gradient (17 ml/frs.) to afford catechin **1** (subfrs.69-79, 70 mg) and gallocatechin **2** (subfrs.32-41, 33 mg). Frs. 3 (3900-4600 ml,1.6 g) was separated on MCI-gel with the same gradient as above to afford (subfrs.76-89, 9 mg) epicatechin-(4β→8)-catechin (**7**) and (subfrs.61-72, 350 mg) epicatechin-(4β→2)-phloroglucinol (**3**). Fraction 3 (4600-4800 ml, 0.8 g) was separated on MCI-gel to afford (subfrs. 23-31, 220 mg) epigallocatechin- (4β→2) -phloroglucinol (**4**). Epicatechin- 3 - O -gallat- (4β→2) - phloroglucinol (**5**) was isolated from fraction 4 (4800-5200 ml, 0.7g) and after MCI-gel chromatography (subfrs. 71-79, 335 mg). Epigallocatechin-3-O-gallat-(4β→2)-phloroglucinol (**6**) was achieved from fraction 5 (5200-5700 ml, 0.4 g) followed by MCI-gel chromatography as described above (subfrs. 59-67, 122 mg). Epicatechin-3-O-gallat-(4 β→8)-epicatechin-3-O-gallat-(4β→2)-phloroglucinol (**8**) was achieved from fraction 6 (6100-6700 ml, 0.34 g) followed by MCI-gel chromatography as described above (subfrs. 71-76, 43 mg). Compounds **1-7** were identified after

acetylation by their physical data (NMR,MS,CD) and compared with those of authentic samples and published values [28-33].

Epicatechin-(4β→2)-phloroglucinol peracetate (3a)

White amorphous powder; ^{1}H-NMR ($CDCl_3$, 400 MHz, $CDCl_3$): δ 1.61-2.33 ppm (3H, *all s,* -OAc), 4.45 (1H, d, *J* 2.3 Hz, H-4C), 5.11 (1H, *dd*, H-3C), 5.11 (1H, *brs*, H-2C), 6.59 (1H, *d, J 2.3* Hz, H-6A), 6.75 (1H, *d, J 2.2* Hz, H-8A), 6.82 (1H, *d, J 2.4* Hz, H-4" or H-6"D), 6.96 (1H, *d, 2.4* Hz, H-6" or H-4"D), 7.16 (1H, *d, J 8.4* Hz, H-5'B), 7.22 (1H, *dd, J* 2.0/8.4 MHz, H-6'B), 7.32 (1H, *d, J* 2.0 Hz, H-2'B). MALDI-TOF-MS (**5a**): *m/z:* 772 ([M + Na]$^+$). $[\theta]_{236nm}$ + 6577, $[\theta]_{277nm}$ +4279.

Epigallocatechin-(4β→2)-phloroglucinol peracetate (4a)

White amorphous powder; ^{1}H-NMR ($CDCl_3$, 400 MHz, $CDCl_3$): δ 1.62-2.34 ppm (3H, *all s,* -OAc), 4.46(1H, d, *J* 2.3 Hz, H-4C), 5.09 (1H, *dd*, H-3C), 5.46 (1H, *brs*, H-2C), 6.59 (1H, *d, J 2.3* Hz, H-6A), 6.74 (1H, *d, J 2.3* Hz, H-8A), 6.81 (1H, *d, J 2.4* Hz, H-4" or H-6"D), 6.96 (1H, *d, 2.4* Hz, H-6" or H-4"D), 7.17 (2H, *s*, H-2`/H-6`B). MALDI-TOF-MS (**6a**): m/z: 830 ([M + Na]$^+$). $[\theta]_{231nm}$ + 6188, $[\theta]_{280nm}$ +3991.

Epicatechin-3-O-gallat-(4β→2)-phloroglucinol peracetate (5a)

White amorphous powder; ^{1}H-NMR ($CDCl_3$, 400 MHz, $CDCl_3$): δ 1.60-2.35 ppm (3H, *all s,* -OAc), 4.50 (1H, d, *J* 2.1 Hz, H-4C), 5.34 (1H, *dd*, H-3C), 5.47 (1H, *brs*, H-2C), 6.61 (1H, *d, J 2.2* Hz, H-6A), 6.74 (1H, *d, J 2.2* Hz, H-8A), 6.79 (1H, *d, J 2.2* Hz, H-4'"E or H-6'"E), 6.92 (1H, *d, 2.2* Hz, H-4'"E or H-6'"E), 7.07 (1H, *dd*, H-6'B), 7.19 (1H, *d*, H-2'B), 7.20 (1H, *d*, H-5'B), 7.47 (2H, s, H-2"/H6"D). MALDI-TOF-MS (**7a**): m/z: 1008 ([M + Na]$^+$). $[\theta]_{233nm}$ + 3190, $[\theta]_{276nm}$ -534.

Epigallocatechin-3-O-gallat-(4β→2)-phloroglucinol peracetate (6a)

Pink amorphous powder; ^{1}H-NMR ($CDCl_3$, 400 MHz, $CDCl_3$): δ 1.57-2.30 ppm (3H, *all s,* -OAc), 4.50 (1H, d, *J* 2.3 Hz, H-4C), 5.34 (1H, *dd*, H-3C), 5.47 (1H, *brs*, H-2C), 6.61 (1H, *d, J 2.3* Hz, H-6A), 6.74 (1H, *d, J 2.3* Hz, H-8A), 6.78 (1H, *d, J 2.4* Hz, H-4'" or H-6'"E), 6.92 (1H, *d, 2.4* Hz, H-4'" or H-6'"E), 7.18 (2H, *s*, H-2`/H-6`B), 7.47 (2H, *s*, H-2"/H6"D). MALDI-TOF-MS (**8a**): m/z: 1066 ([M + Na]$^+$). $[\theta]_{236nm}$ +3157, $[\theta]_{273nm}$ -301.

Epicatechin-3-O-gallat-(4 β→8)-epicatechin-3-O-gallat-(4β→2)-phloroglucinol peracetate (8a)

White amorphous powder; ^{1}H-NMR ($CDCl_3$, 400 MHz, $CDCl_3$): δ 1.52-2.26 ppm (3H, *all s,* -OAc), 4.54 (1H, *d, J 1.7* Hz, H-4F), 4.88 (1H, *brs*, H-4C), 5.29 (1H, *brs*, H-2C), 5.48 (1H, *m*, H-3F), 5.65 (1H, *m*, H-3C), 5.68 (1H, *brs*, H-2F), 6.58 (1H, *s*, H-6D), 6.66 (1H, *d, J 2.4* Hz, H-6A), 6.72 (1H, *d, J 2.4* Hz, H-8A), 6.84 (1H, d, *J* 2.3 Hz, H-4''' or H-6'''I), 6.79-7.21 (2 x B and E ring protons), 6.98 (1H, d, *J* 2.3 Hz, H-4''' or H-6'''I), 7.33 (2H, *s*, H-2"/H-6"G), 7.43 (2H, *s*, H-2"/6"H). MALDI-TOF-MS (**8a**): m/z: 1744 ($[M + Na]^+$). $[\theta]_{230nm}$ + 2277.

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