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Isolation of two new bioactive proanthocyanidins from *Cistus salvifolius* herb extract

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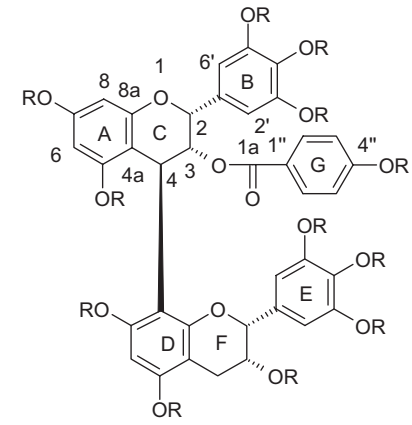
Two new proanthocyanidins, epigallocatechin-3-*O*-*p*-hydroxybenzoate-(4β→8)-epigallocatechin (**1**) and epigallocatechin-3-*O*-*p*-hydroxybenzoate-(4β→8)-epigallocatechin-3-*O*-gallate (**2**) in addition to the known compound epigallocatechin-(4β→6)-epigallocatechin-3-*O*-gallate (**3**), were isolated from the air-dried herb of *Cistus salvifolius*. The chemical structures were determined on the basis of 1D-and 2D-NMR-spectra (HSQC, HMBC) of their peracetylated derivatives, MALDI-TOF-mass spectra, and by acid-catalysed degradation with phloroglucinol. The isolated compounds **1**–**3** and the water extract of *C. salvifolius* herb were tested for their inhibitory activities against COX-1 and COX-2. Compound **2** showed the strongest inhibitory effect on COX-2 followed by compound **3**, compound **1** and the water extract, while compounds **1**–**3** exhibited moderate *in vitro* inhibition against COX-1.

1. Introduction

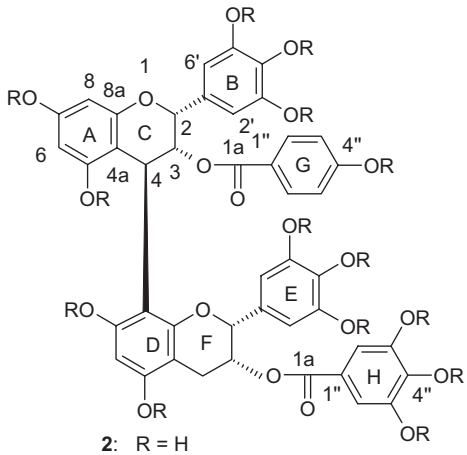
The genus *Cistus* (Cistaceae) comprises about 20 shrub species found in wide areas throughout the whole Mediterranean region (Comandini et al. 2006). *Cistus* species are used as anti-diarrheic, as general remedies in folk medicine for the treatment of various skin diseases, and as anti inflammatory agents (Attaguile et al. 2000; Petereit 1992). Furthermore, a specific polyphenol containing *Cistus incanus* extract (CYSTUS052) demonstrated antiviral activity against influenza A virus infections (Droebner et al. 2007; Ehrhardt et al. 2007). In Jordan, a tea prepared from *Cistus salvifolius* L. herb has traditionally been used for the treatment of gout (Al-Khalil 1995). Pharmacological investigations on traditional Turkish plants have shown that *C. salvifolius* is highly effective for the treatment of ulcer (Yesilada et al. 1999). The ethyl acetate-soluble fraction from the aqueous acetone extract was found to contain the following flavan-3-ols and dimeric prodelphinidins: catechin, gallocatechin, epicatechin, epigallocatechin, epicatechin-3-*O*-gallate, epigallocatechin-3-*O*-gallate, epigallocatechin-3-*O*-*p*-hydroxybenzoate, epigallocatechin-(4β→8)-epigallocatechin, epigallocatechin-3-*O*-gallate-(4β→8)-epigallocatechin, and epigallocatechin-(4β→6)-epigallocatechin-3-*O*-gallate (Danne et al. 1994). In a recent study, a higher oligomeric proanthocyanidin containing fraction isolated from the title plant exhibited the strongest antioxidant activity of all isolated fractions (Qa'dan et al. 2006). In continuation of our phytochemical and pharmacological studies on *Cistus* species, we report here on the isolation, structure elucidation and testing of new polyphenolic compounds for their inhibitory activities against COX-1 and COX-2.

2. Investigations, results and discussion

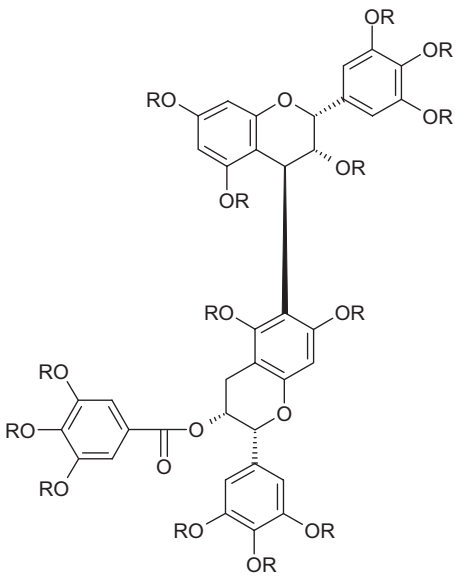
Compound **1** showed a prominent quasi-molecular ion peak at *m/z* 1257 (M + Na(+ in the MALDI-TOF-MS of its peracetate (**1a**), which suggests a B-type dimeric prodelpinidin with a *p*-hydroxybenzoic acid acylation. The ^{1}HNMR of **1a** in $CDCl_3$ (600 MHz) was similar to that of the analogous proanthocyanidin epigallocatechin-(4β→8)-epigallocatechin-3-*O*-*p*-hydroxybenzoate peracetate (De Mello et al. 1996). The ^{1}H-NMR of **1a** gave two two-proton singlets at δ 6.90 and 7.22 ppm typical for pyrogallol-type-B rings of the constituent flavan-3-ol units. The heterocyclic coupling constants ($J_{2,3} < 2$ MHz) confirmed the relative 2,3-*cis* configuration of the "upper" and "lower" constituent units (Fletcher et al. 1977). The acyl substituent was identified from the AA'BB' type aromatic signal set at δ 7.11 and 7.88 ppm, which indicate the equivalent protons of a *p*-hydroxybenzoyl group. The ^3J cross peak of H-3 (C) to the carboxyl group was the direct proof for the point of attachment of the acyl substituent on C-3 position of the upper flavan-3-ol unit. The location of the interflavanoid linkage was recognized for **1a** by long-range correlation (HMBC) of the H-4 (C) with the C-8a (D) (Balas and Vercauteren 1994). This key correlation indicates that the flavan-3-ol units are C-4/C-8 linked. The correlation between H-8 (A) and H-4 (C) to the C-8a (A) confirmed the "upper" flavan-3-ol unit as epigallocatechin-3-*O*-*p*-hydroxybenzoate. Compound **1** gave epigallocatechin-3-*O*-*p*-hydroxybenzoate-(4β→2)-phloroglucinol as the main degradation product, and epigallocatechin as releasing terminal flavan-3-ol (Foo and Karchesy 1989). These degradation products were identified by NMR-spectroscopy and compared with authentic compounds. The intense positive Cotton effect at 210–240 nm in the CD spectrum of **1a** is in line with the 4β-linkage, and thus a 4*R* absolute configuration (Barrett et al. 1979; Botha et al. 1981). In conjunction with the optical rotation $[\alpha]_D^{20} = -47.3^\circ$ (*C* 0.18, MeOH), compound **1** was identified as epigallocatechin-3-*O*-*p*-hydroxybenzoate-(4β→8)-epigallocatechin.



1: R = H
1a: R = Acetyl



2: R = H
2a: R = Acetyl



3: R = H
3a: R = Acetyl

Compound **2** showed a prominent quasi-molecular ion peak at *m/z* 1493 (M + Na(+ in the MALDI-TOF-MS of its peracetate (**2a**), indicating an acylated prodelphinidin dimer. The ^{1}H-NMR of **2a** in $CDCl_3$ (600 MHz) was similar to that of the analogous proanthocyanidin epigallocatechin-3-*O*-gallate-(4β→8)-epigallocatechin-3-*O*-gallate-peracetate (De Mello et al. 1996). The ^{1}H NMR of **2a** gave two two-proton singlets at (6.82 and 7.07 ppm typical for pyrogallol-type-B rings

Table: IC_{50} values of isolated compounds as inhibitors of COX-1 and COX-2[a]

	COX-1 [μM]	COX-2 [μM]
WE	63 ± 1.0	44.7 ± 0.8
1	78.9 ± 1.7	37.6 ± 3.2
2	65.2 ± 0.9	12.3 ± 0.3
3	73.5 ± 2.1	29.3 ± 1.1
ASA	21.3 ± 0.4	17.5 ± 0.3

[a] Values are means ± data obtained from three different assays. WE water extract

of the constituent flavan-3-ol units. The heterocyclic coupling constants ($J_{2,3} < 2$ MHz) confirmed the relative 2,3-*cis* configuration of the "upper" and "lower" constituent units (Fletcher et al. 1977). The identity of the acyl substituents was obvious from an AA'BB' type aromatic signal set at δ 7.18 and 7.90 ppm and a sharp low-field two-proton singlet (*δ* 7.72 ppm), which indicate the equivalent protons of a *p*-hydroxybenzoyl group and a galloyl moiety, respectively. The long-range correlation signals between the H-3 (C) and H-3 (F) with the corresponding carboxyl functions of the attached acyl moieties were the direct proof for the point of attachment at C-3 position of both units. The location of the interflavanoid linkage was recognized for **2a** by long-range correlation (HMBC) of the H-4 (C) with the C-8a (D) (Balas and Vercauteren 1994). This key correlation indicates that the flavan-3-ol units are C-4/C-8 linked. The correlation between H-8 (A) and H-4 (C) to the C-8a (A) confirmed the "upper" flavan-3-ol unit as epigallocatechin-3-*O*-*p*-hydroxybenzoate. Compound **2** gave epigallocatechin-3-*O*-*p*-hydroxybenzoate-(4β→2)-phloroglucinol as the main degradation product, and epigallocatechin-3-*O*-gallate as releasing terminal flavan-3-ol (Foo and Karchesy 1989). These degradation products were identified by NMR-spectroscopy and in comparison with authentic compounds. The intense positive Cotton effect at 210–240 nm in the CD spectrum of **2a** is in line with the 4β-linkage, and thus a 4*R* absolute configuration (Barrett et al. 1979; Botha et al. 1981). In conjunction with the optical rotation $[\alpha]_D^{20} = -33.6^\circ$ (*C* 0.15, MeOH), compound **2** was identified as epigallocatechin-3-*O*-*p*-hydroxybenzoate-(4β→8)-epigallocatechin-3-*O*-gallate.

The structure of compound **3** was identified on the basis of 1D- and 2D-NMR (HSQC, HMBC) experiments of its peracetylated derivatives. Comparison of the data with authentic samples from earlier work and published values, identified this compound as epigallocatechin-(4β→6)-epigallocatechin-3-*O*-gallate (De Mello et al. 1996). To the best of our knowledge, compounds **1** and **2** are described here for the first time.

The *Cistus* water extract (WE) and the isolated compounds were tested for their *in vitro* inhibitory activities against cyclooxygenase (COX-I and COX-2, Table 1). The WE and the pure compounds **1**–**3** were found to have potent COX-2 inhibitory effects, with IC_{50} values of 44.7 μM, 37.6 μM, 12.3 μM and 29.3 μM, respectively; the WE and all the isolated compounds (**1**–**3**) exhibited moderate inhibition against COX-1 (Table). The new compound epigallocatechin-3-*O*-*p*-hydroxybenzoate-(4β→8)-epigallocatechin-3-*O*-gallate (**1**) showed higher COX-2 inhibitory activity (IC_{50} = 12.3 μM) than the positive control acetyl salicylic acid (ASA, IC_{50} = 17.5 μM). The presence of a *p*-hydroxybenzoyl moiety in addition to the galloylated moiety, and two trihydroxylated B-rings in the isolated new compound **2**, and its relative higher molecular weight might be responsible for the potent inhibitory activity against COX-2 (Seeram et al. 2003). The water extract showed relatively high inhibition (Table), possibly because it contains in

addition to the known tannins (Danne et al. 1994; Qa'dan et al. 2006) also other compounds like flavonols and phloroglucinol glucosides (Danne et al. 1994) which were reported to have anti-inflammatory effects (Clavin et al. 2007). Further studies are necessary to explore in more depth the anti-inflammatory potency of compound **2**.

In conclusion, the two new isolated proanthocyanidins (Fig. 1) showed great similarity in the chemical structure to the flavan-3-ols and oligomeric proanthocyanidins isolated previously from the title plant (Danne et al. 1994; Qa'dan et al. 2006) in the predominance of 2,3-*cis*-configuration, 3',4',5'-trihydroxylated B-rings and the presence of galloylated units.

3. Experimental

3.1. General experimental procedures

NMR spectra were recorded in $CDCl_3$ with a a Bruker AM 600 instrument. Chemical shifts were recorded relative to $CHCl_3$. 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; acceleration voltage 16 kV; 1 m drift length; data logging with LeCroy9450A; sampling time 2.5 ns, expected mass accuracy, +/– 0.1%; 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) with the mobile phase EtOAc/HCOOH/H_2O (18:1:1). Compounds were visualized as red spots by spraying with vanillin/HCl-reagent. Optical rotation ([α]) was measured using a Perkin-Elmer polarimeter 241.

3.2. Chemicals and reagents

COX-1 and COX-2 were purchased from Sigma (Munich, Germany). 1-^{14}C-labeled arachidonic acid (>200 μCi (370 kBq), NEN) was purchased from New England Nuclear Co. (Boston, USA). Other chemicals and reagents were purchased from Roth Chemicals (Dubai, UAE).

3.3. Plant material

Cistus salvifolius L. was collected in El Majdal (Jordan; 04/2006) and identified in comparison with authentic *Cistus salvifolius* obtained from the Botanical Garden, University of Jordan (Amman). A voucher specimen is deposited at the Petra University, Amman-Jordan under PUAM19.

3.4. Extraction and isolation

Air-dried material (4 kg) was exhaustively extracted with 30 L hot water (40 °C) and the combined extracts were evaporated *in vacuo* to dryness (534 g). A portion (100 g) of the water-extract was applied to CC on Sephadex LH-20 (55 × 900 mm) and successively eluted with 5 L EtOH-H_2O and 20 L MeOH-H_2O (1:1) to afford 10 fractions. Fraction 8 (17500 - 18200 ml, 560 mg) obtained from Sephadex LH-20 column was subjected to chromatography on MCI gel CHP 20P (25 × 450 mm) with a 10–60% MeOH (3 L) linear gradient (16 ml/subfraction) to afford an amorphous white powder (compound **3,** subfractions 16 - 23, 15.5 mg). Compound **3** was identified after acetylation by its physical data (NMR, MS, CD) and by comparison with an authentic sample and published values (De Mello et al. 1996).

3.5. Epigallocatechin-3-O-p-hydroxybenzoate-(4β→ 8)-epigallocatechin

(**1**): Fraction 7 (16700–17500 ml, 760 mg) obtained from Sephadex LH-20 column was subjected to chromatography on MCI gel CHP 20P (25 × 450 mm) with a 10 - 60% MeOH (3 L) linear gradient (compound **1**, 16 ml/subfraction) to afford an amorphous white powder (compound subfractions 33 - 38, 41 mg); Acetylation yielded epigallocatechin-3-*O*-p-hydroxybenzoate-(4β → 8)-epigallocatechin-*peracetate* (**1a**). $[\alpha]_D^{20}$ = + 47,3° (*c* 0.18, MeOH); MALDI-TOF-MS: [M + Na] $^+$ *m/z* 1257. CD $[\theta]_{293}$ + 2879, $[\theta]_{274}$ –12134, $[\theta]_{237}$ + 131455, $[\theta]_{216}$ – 15685, $[\theta]_{210}$ + 171. Degradation of 12 mg **1** with 15 mg phloroglucinol in 2.5 mL 1% ethanolic HCl yielded 5 mg epigallocatechin-3-*O*-*p*-hydroxybenzoate-(4β→2)-phloroglucinol and 3.7 mg of the intact epigallocatechin-3-*O*-gallate 5.5 mg, which were purified using a Sephadex LH-20 column (60 × 25 mm) with first 500 ml EtOH-MeOH (1:1), then 400 mL MeOH as eluent and identified as peracetates with NMR and MALDI-TOF-MS experiments. ^{1}H-NMR ($CDCl_3$, 600 MHz): δ 1.88–2.28 [m, aliphatic and aromatic OAc], δ 2.91 [m, H-4ax (F) and H-4eq (F)], δ 4.53 [d, *J* = 1.9 Hz, H-4 (C)], δ 4.66 [brs, H-2 (F)], δ 5.53 [brs, H-2 (C)], δ 5.59 [m, H-3 (C)], δ 5.75 [m, H-3 (F)], δ 6.30 [d, *J* = 2.3 Hz, H-8 (A)], δ 6.33 [d, *J* = 2.3 Hz, H-6 (A)], δ 6.71 [s, H-6 (D)], δ 6.90 [s, H-2'/H-6' (E)], δ 7.11 [d, *J* = 8.7 Hz, H-3"/H-5" (G)], δ 7.22 [s, H-2'/H-6' (H)], 7.88 [d, *J* = 8.7 Hz, H-2"/H-6" (G)]. ^{13}C-NMR ($CDCl_3$, 150 MHz): δ 29.3 [C-4 (F)], δ 36.5 [C-4 (C)], δ 67.7 [C-3 (F)], δ 72.4 [C-3 (C)], δ 74.7 [C2 (C)], δ 76.6 [C-2 (F)], δ 108.1 [C-6 or C-8 (A)], δ 108.5 [C-6 or C-8 (A)], δ 111.3 [C-8 (D)], δ 121.6 [C-2"/C-6" (G)], δ 127.1 [C-1" (G)], δ 138.8 [C-4" (G)], δ 142.3 [C-3"/C5" (G)], δ 163.6 [Carboxyl (G)]. G represents the ^{1}H/^{13}C signals of the attached hydroxybenzoyl moiety.

3.6. Epigallocatechin-3-O-p-hydroxybenzoate-(4β→8) epigallocatechin-3-O-gallate (2)

Fraction 8 (17500 - 18200 ml, 560 mg) achieved from Sephadex LH-20 column was subjected to chromatography on MCI gel CHP 20P (25 × 450 mm) with a 10 - 60% (3 L) MeOH linear gradient (16 ml/subfraction) to afford an amorphous white powder (compound **2**, subfractions 43–47, 33 mg); Acetylation yielded epigallocatechin-3-*O*-p-hydroxybenzoate-(4β→ 8)-epigallocatechin-3-O-gallate *-peracetate* (**1a**). $[\alpha]_D^{20}$ = + 46,65° (*c* 0.19, MeOH); MALDI-TOF-MS: [M + Na] $^+$ *m/z* 1377. CD $[\theta]_{295}$ + 3029, $[\theta]_{278}$ –10337, $[\theta]_{234}$ + 172395, $[\theta]_{216}$–16155, $[\theta]_{210}$ + 91. Degradation of 18 mg **1** with 15 mg phloroglucinol in 2.5 mL 1% ethanolic HCl yielded 7 mg epigallocatechin-3-*O*-*p*-hydroxybenzoate-(4β→2)-phloroglucinol and 5.5 mg of the intact epigallocatechin-3-*O*-gallate 5.5 mg, which were purified using a Sephadex LH-20 column (60 × 25 mm) with first 500 ml EtOH-MeOH (1:1), then 400 mL MeOH as eluent and identified as peracetates with NMR and MALDI-TOF-MS experiments. ^{1}HNMR ($CDCl_3$, 600 MHz, H_R = assignment of rotameric signals): δ 2.07–2.31 [m, aliphatic and aromatic OAc], δ 2.78 [d, *J* = 16.9 Hz, H_R-4a (F)], δ 2.93 [d, *J* = 16.9 Hz, H-4a (F)], δ 3.09 [s, H-4b (F)], δ 4.34 [s, H_R-4 (C)], δ 4.51 [s, H-4 (C)], δ 5.29 [s, H-2 (F)], δ 5.43 [s, H-2 (C)], δ 5.53 [s, H-3 (C)], δ 5.65 [m, H_R-3 (F)], δ 5.70 [m, H-3 (F)], δ 6.70 [d, *J* = 2.2 Hz, H-6 or H-8 (A)], δ 6.79 [d, *J* = 2.2 Hz, H_R-6 or H_R-8 (A)], δ 6.82 [d, *J* = 2.2 Hz, H-6 or H-8 (A)], δ 6.76 [s, H-8 (D)], δ 6.87 [s, H_R-8 (D)], δ 7.09–7.41 [proton signals of the rings B and E], δ 7.10 [d, *J* = 8.8 Hz, H_R-3" H_R-5" (H)], δ 7.14 [d, *J* = 8.8 Hz, H-3" H-5" (H)], δ 7.51 [s, H-2" H-6" (G)], δ 7.54 [s, H_R-2" H_R-6" (G)], δ 7.86 [d, *J* = 8.8 Hz, H_R-2" H_R-6" (H)], δ 7.92 [d, *J* = 8.8 Hz, H-2" H-6" (H)]. ^{13}C-NMR ($CDCl_3$, 150 MHz, C_R = assignment of rotameric signals): δ 26.52 [C-4 (F)], δ 35.15 [C-4 (C)], δ 67.45 [C-3 (F)], δ 67.53 [C_R-3 (F)], δ 72.35 [C-3 (C)], δ 74.97 [C_R-2 (C)], δ 74.02 [C-2 (C)], δ 76.88 [C-2 (F)], δ 107.33 [C-6 or C-8 (A)], δ 107.35 [C_R-6 or C_R-8 (A)], δ 108.88 [C-6 or C-8 (A)], δ 109.80 [C_R-8 (D)], δ 110.67 [C-8 (D)], δ 121.60 [C-3" C-5" (H)], δ 122.24 [C-2" C-6" (G)], δ 127.48 [C-1" (G)], δ 131.50 [C-2" C-6" (H)], δ 139.00 [C-4" (G)], δ 143.52 [C-3" C5" (G)], δ 154.14 [C-4" (H)], δ 162.93 [Carboxyl (G)], δ 165.44 [Carboxyl (H)]. G represents the ^{1}H/^{13}C signals of the attached galloyl moiety, H represents the ^{1}H/^{13}C signals of the attached hydroxybenzoyl moiety.

3.7. Effect on cyclooxygenase –1 and –2

The effect on cyclooxygenase-1 and –2 (COX-1 and COX-2) was determined by measuring PGE_2 production. The reaction mixtures were prepared in tris(hydroxymethyl)aminomethane-HCl buffer (pH 8.0), containing glutathione (350 μM), epinephrine (350 μM), hematin (1.5 μM), enzyme (COX-1 or COX-2, 50 μl) and various concentrations of the water extract (WE) or isolated compounds. 1–^{14}C arachidonic acid (10 μl) was added to start the reaction. The mixture was first incubated for 30 min at 37 °C, then the reaction was terminated by adding the reaction mixture (20 μl) to 30 μM indomethacin (200 μl). Arachidonic acid and its radio-labeled metabolites were separated and determined by reversed-phase HPLC using a Berthold radioactivity monitor (Pharma Tech R&D, Amman, Jordan). Inhibition refers to the reduction of PGE_2 formation, in comparison to a blank run without inhibitor. ASA was used as a positive control. The results are means of three independent experiments.

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