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PRODELPHINIDINS FROM *RIBES NIGRUM*

M. TITS, L. ANGENOT, P. POUKENS, R. WARIN* and Y. DIERCKXSENS†

Institut de Pharmacie, Université de Liège, rue Fusch, 5, B-4000 Liège, Belgium; *Institut de Chimie, Université de Liège, Campus du Sart Tilman, B-4000 Liège, Belgium; †L. P. H. DOLISOS, rue Carli, 3, B-1140 Brussels, Belgium

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Key Word Index—*Ribes nigrum*; Grossulariaceae; blackcurrant leaves; proanthocyanidins; prodelphinidins.

Abstract—*Ribes nigrum* leaves yielded three anti-inflammatory prodelphinidins. These compounds were identified and characterized; two known prodelphinidin dimers gallocatechin-(4α→8)-epigallocatechin and gallocatechin-(4α→8)-gallocatechin found together for the first time and a new prodelphinidin trimer gallocatechin-(4α→8)-gallocatechin-(4α→8)-gallocatechin.

INTRODUCTION

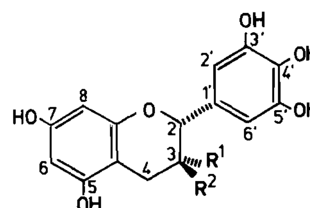
An infusion of blackcurrant leaves, *Ribes nigrum* L. is traditionally used in Europe for the treatment of rheumatic disease. Diuretic and antiinflammatory properties may lie at the basis of this ethnopharmacological reputation [1]. In screening a number of such medicinal plants, we have observed that ethanol or aqueous acetone extracts of *R. nigrum* at 50 mg kg^{-1} i.p. significantly inhibited carrageenan rat-paw oedema (M. Tits, personal communication). The activity has since been confirmed in another laboratory [2]. We have now shown that the flavonoids and phenolic acids previously found in this plant [3, 4] are not responsible for this strong activity. Instead, the most active fractions contained proanthocyanidins, including polymers known to be present in several species of *Ribes* (*R. grossularia*, *R. nigrum*, *R. rubrum* and *R. sanguineum*) [5–7]. We report here on the isolation and characterization of three bioactive molecules from *R. nigrum*: two known prodelphinidin dimers and a new prodelphinidin trimer.

RESULTS AND DISCUSSION

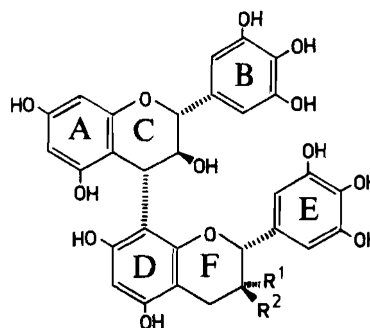
The aqueous soluble fraction obtained by aqueous acetone extraction of *R. nigrum* leaves was fractionated by a combination of medium pressure liquid chromatography (MPLC) on RP8 and Sephadex LH20. The initial fractions contained monomers, principally gallocatechin (**1**), epigallocatechin (**2**) and latter fractions eluted proanthocyanidins with an increasing degree of polymerization.

According to the FAB mass spectra, the major dimers have the same M_r. They were identified as gallocatechin-(4α→8)-epigallocatechin (**4**), isolated for the first time from *Ribes sanguineum* [8] and recently also found in Oolong tea [9] and *Ostrya virginiana* [10] and its C-3F isomer: gallocatechin-(4α→8)-gallocatechin (**3**) previously obtained from *Quercus dentata* [11], *Mallotus japonicus* [12], *Corylus avellana* [10] and *Cistus incanus* [13]. It is the first time that these two isomers have been found to occur together in the same plant and their separation was very difficult. Their structure was derived from the analysis of the ^1H NMR [8, 10, 13] and CD [10, 13, 14] spectra of their acetates as also the analysis of the ^{13}C NMR [11] and IR spectra [15]. Comparison of the ^{13}C NMR spectrum of **4** with those of **3** described by Sun [11] showed principally differences in the C-2 and C-3 chemical shifts, indicating that the terminal unit is 2,3 *cis* in **4** (δC-2 78.6 and δC-3 66.4) and 2,3 *trans* in **3** (δC-2 83.1 and δC-3 68.2).

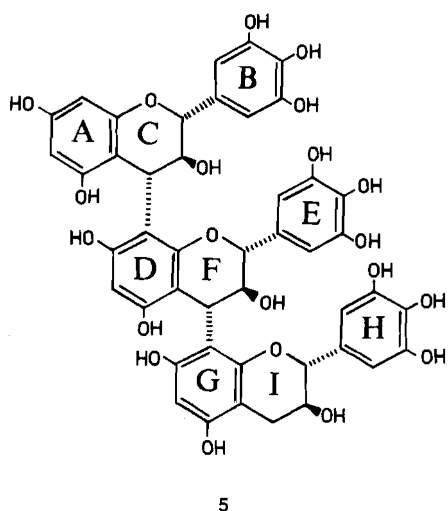
The M_r of the third major procyanidin (**5**) was established from the FAB mass spectrum which gave a molecular ion at m/z 914. It is interesting to point out that the expected quasi-molecular ion $[M + H]^+$ at m/z 915



1 R^1 = H R^2 = OH
2 R^1 = OH R^2 = H



3 R^1 = H R^2 = OH
4 R^1 = OH R^2 = H



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could not be observed on the spectrum of the trimer dissolved in glycerol. It is, however, known that the neutral sample molecule M can also be desorbed from the matrix [18].

Examination of the high-field aromatic region of the ^{13}C NMR spectrum revealed signals at δ108, identified with C-2′ and C-6′ of three pyrogallol rings (B, E and H) and at δ145–146 corresponding to C-5′ and C-3′ of the same rings. No signals were detected at δ143 and 115 which would correspond to C-5′ and C-3′ of procyanidins. Therefore, this trimer only contains prodelphinidin units. Examination of the δ30–90 region which includes the shifts of the heterocyclic ring carbons, showed the relative downfield position of C-2, between δ81 and 84, indicating that the three flavan units possessed the same 2,3-*trans* configuration. The absence of a signal about δ78 supports this stereochemistry.

Analysis of the following coupling constants derived from the ^1H NMR and ^1H COSY spectra of the acetate trimer [upper unit (C ring) and middle unit (F ring) (J_{2-3} = 10 Hz and J_{3-4} = 9 Hz)] indicated a 2,3-*trans* and 3,4-*trans* orientation, respectively, for these rings. The intense negative Cotton effect confirmed the 4*S*-configuration where the two rings are linked [14]. The smaller chemical shift difference between H-2 and H-3 in the terminal unit required a 2,3-*trans* stereochemistry and was in agreement with a (4→8) linkage between the middle and the lower units [16]. Further corroborative evidence for this lower unit was obtained by acid degradation (HCl 0.1 M) which yielded gallocatechin. The above analysis suggested a trigallocatechin constitution with the upper ring linked to either C-6 or C-8.

Analysis of the aromatic region of the ^1H NMR spectrum indicated for the A ring a *meta*-coupled doublet attributable to the H-6 and the H-8 at δ6.36 and 6.54 (J_{6-8} = 2.3 Hz). The chemical shifts for rings B, E and H, which are of the pyrogallol type, were at δ6.65, 6.68 and 6.96. These shifts indicated the mode of linkage in acetate prodelphinidins. Indeed, in acetate (4→8) prodelphinidins, we observed a consistent shielding relative to those of acetate (4→6) prodelphinidins, where the shifts of H-2′ and H-6′ are downfield (< 7 ppm). Petereit *et al.* [13] explained this difference by the anisotropy of the 3-acetoxy functions and the respective aromatic rings of the constituent units.

The structure of the novel trimer is thus gallocatechin-(4α→8)gallocatechin-(4α→8)-gallocatechin (**5**). To the best of our knowledge, it is the first example of a natural product possessing three unsubstituted gallocatechin units.

EXPERIMENTAL

^1H and ^{13}C NMR spectra were measured at 400 MHz, in $CDCl_3$ and Me_2CO-d_6–H_2O (1:1), respectively; chemical shifts are given in δ (ppm) scale relative to TMS. MS were recorded using FAB positive mode system; samples were dissolved in glycerol matrix. CD data were obtained in MeOH. TLC was performed on silica gel 60 F_{254} (Merck) with EtOAc–H_2O–HCO_2H–HOAc (70:20:3:2) upper phase (solvent system A) and spots were visualized by spraying with vanillin–HCl reagent. Prep. TLC plates (0.5 mm) were prepared with silica gel 60 PF_{254} Merck and acetates purified with toluene–Me_2CO (7:3) (solvent system B). Acetylations were performed in Ac_2O–pyridine (1:1) at ambient temp.

Conversion of proanthocyanidins into anthocyanidins. The proanthocyanidin (*ca* 1 mg) was refluxed with 5% HCl in EtOH for 1 hr. The reaction mixt. was subsequently chromatographed on cellulose (cellulose F, 0.1 mm, Merck) in HCO_2H–HCl–H_2O (10:1:3) (solvent system C) with delphinidin and cyanidin as ref. substances.

Identification of lower terminal flavan-3-ol unit. The proanthocyanidin (*ca* 1 mg) was treated with 0.1 M ethanolic HCl (2 ml) at 60° for 15 min. The lower terminal flavan-3-ol was liberated and detected by TLC on cellulose in H_2O–dioxan (10:1) (solvent system D).

Plant material. Blackcurrant leaves were purchased from a Belgian medicinal plant drug outlet (Denolin, Braine-l'Alleud). They were compared to leaves of *Ribes nigrum* L. collected in the garden of one of us, located in Flémalle (Les Awirs). Both samples were similar and identified according to the description of L. Nihoul [17]. A voucher specimen is deposited in the herbarium of the Pharmaceutical Institute, University of Liège.

Extraction, isolation and identification of compounds. The leaves (1 kg) were powdered mechanically and then extracted at room temp. with 70% Me_2CO aq. The Me_2CO was removed by evapn under red. pres. (*ca* 40°). The resulting aq. soln was freeze-dried (240 g). A portion of this material (4.2 g × 4) was then fractionated by MPLC on RP8 with H_2O–Me_2CO (9:1) to afford 3 frs: I (carbohydrates), II (proanthocyanidins monomers and oligomers 4.1 g) and III (a flavonoid glycoside and polymers) and then with H_2O–Me_2CO (1:1) containing other flavonoid glycosides and proanthocyanidin polymers with higher M_r. This chromatographic procedure was effective for a clean-cut separation of proanthocyanidins and other compounds of this plant. An EtOH soluble portion of fr. II was then chromatographed on Sephadex LH20 in EtOH (3.2 l) as the first solvent and in EtOH–MeOH (1:1) (2:8 l) as the second. This yielded 10 frs (a–j).

Monomeric flavan-3-ols. Fr. b (67 mg) contained gallocatechin (**1**) and epigallocatechin (**2**). They were identified by TLC on silica gel (system A) and on cellulose (system D) with gallocatechin and epigallocatechin as reference substances. They afforded a red colour immediately on spraying with vanillin–HCl; however, after some hours, epigallocatechin became browner and gallocatechin changed to violet.

Dimeric proanthocyanidins. Dimers were present in frs c–e. Only the major fr. d (85 mg) has now been studied. We identified gallocatechin-(4α→8)-gallocatechin (**3**) and gallocatechin-

(4α→8)-epigallocatechin (**4**). Their M_r was established from the FAB-MS $[M + H]^+$ at m/z 611. Their separation was very difficult and required another chromatography on RP8 using H_2O–Me_2CO (19:1) to obtain the pure compounds: (**4**) 30 mg and (3) 40 mg. It was noted that the two dimers had the same R_f (0.46) on TLC (system A) and that they afforded a red colour immediately on spraying with vanillin–HCl. However, after 24 hr **4** became browner and 3 violet in the same way as corresponding monomers.

Gallocatechin-(4α→8)-gallocatechin (3). Conversion into anthocyanidins, afforded delphinidin and treatment with 0.1 M HCl liberated gallocatechin. IR, ^{13}C NMR: see literature data [11]. CD, ^{1}H NMR of the acetate were identical to those previously reported [10, 13].

Gallocatechin-(4α→8)-epigallocatechin (**4**). Conversion into anthocyanidins, afforded delphinidin and treatment with 0.1 M HCl liberated epigallocatechin. IR: identical to that described in ref. [15]. ^{13}C NMR: δ28.9 (C-4 F), 37.6 (C-4 C), 66.5 (C-3 F), 73.0 (C-3 C), 78.7 (C-2 F), 83.3 (C-2 C), 95.6 (C-8 A), 97.2 (C-6 D), 97.3 (C-6 A), 99.1 (C-4a D), 101.1 (C-4a A), 107.4 (C-8 D), other A and D rings carbons (154.2, 154.3 154.6, 155.6, 156.5 157.6), 106.6 (C-2′E, C-6′E), 108.4 (C-2′B, C-6′B), 130.9 and 131.7 (C-1′B, E), 133.5 (C-4′B, E), 145.8 (C-3′, C-5′B, E). CD, ^{1}H NMR of the acetate: see literature data [8, 10].

Trimeric prodelphinidin: *gallocatechin-(4α→8)-gallocatechin-(4α→8)-gallocatechin* (**5**). Fr. g (75 mg) afforded the novel trimer (**5**) TLC R_f 0.16 (system A) coloured in red with vanillin–HCl. FAB-MS m/z 914. Conversion into anthocyanidins gave delphinidin and treatment with 0.1 M HCl liberated gallocatechin. ^{13}C NMR: δ37.6 and 37.8 (C-4C, C-4 F), 67.9 (C-3I), 72.8 (C-3C, C-3 F), 81 to 84.5 (C-2C, F, I), 106.1 to 109 (C-2′B, E, H; C-6′B, E, H and C-8D, G), 130.9 and 131.3 (C-1′B, E, H), 133.4 (C-4′B, E, H), 145.3 to 145.9 (C-3′B, E, H and C-5′B, E, H), 154.3 to 157.5 (C-5A, D, G; C-7A, D, G and C-8a A, D, G). IR ν_{max}^{KBr} cm^{-1}: 3600–3000, 1615, 1540, 1515, 1450, 1345, 1205, 1145, 1070, 1030, 825, 730. Acetylation of a portion (50 mg) of the content of this fraction afforded the acetate purified by prep. TLC, R_f 0.18 (system B), yield: 10 mg. ^{1}H NMR and ^{2}D ^{1}H shift correlation spectra (COSY): δ1.94–2.38 (all *s* aliphatic and phenolic OAc), 2.11 and 2.58 (2 × H-4I), 4.20 (*d*, J = 9 Hz, H-4C), 4.57 (*d*, J = 9 Hz, H-4F), 4.68 (*d*, J = 10 Hz, H-2C), 4.78 (*d*, J = 10 Hz, H-2F), 5.18–5.23 (*m*, H-3I, H-2I), 5.39 (*dd*, J = 9 and 10 Hz, H-3 F), 5.57 (*dd*, J = 9 and 10 Hz, H-3 C), 6.36 (*d*, J = 2.3 Hz, H-6 A), 6.54 (*d*, J = 2.3 Hz, H-8 A), 6.58 (*s*, H-6D, G), 6.65, 6.68, 6.96 (3*s*, 6 × H, H-2′B, H-2′E, H-2′H and H-6′B, H-6′E, H-6′H). CD: $\Delta\varepsilon_{275}$ -9.69, $\Delta\varepsilon_{230}$ -27.39. $[\alpha]_D^{20}$ $-140°$ [MeOH–H_2O (1:1); c 0.003].

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