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The structure of cranberry proanthocyanidins which inhibit adherence of uropathogenic P-fimbriated *Escherichia coli* in vitro

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Abstract

Ethyl acetate extracts of Sephadex LH20-purified proanthocyanidins of American cranberry (*Vaccinium macrocarpon* Ait.) exhibited potent biological activity by inhibiting adherence of uropathogenic isolates of P-fimbriated *Escherichia coli* bacteria to cellular surfaces containing α-Gal(1 → 4)β-Gal receptor sequences similar to those on epithelial cells in the urinary tract. The chemical structures of the proanthocyanidins were determined by ^{13}C NMR, electrospray mass spectrometry, matrix-assisted laser absorption time-of-flight mass spectrometry and by acid catalyzed degradation with phloroglucinol. The proanthocyanidin molecules consisted predominantly of epicatechin units with mainly DP of 4 and 5 containing at least one A-type linkage. The procyanidin A2 was the most common terminating unit occurring about four times as frequently as the epicatechin monomer.

Keywords: *Vaccinium macrocarpon*; Ericaceae; Cranberry; *Escherichia coli*; Adherence inhibitors; Urinary tract infection; Proanthocyanidin; A-type; Phloroglucinol adducts; ES-MS; MALDI-TOF

1. Introduction

The American cranberry (*Vaccinium macrocarpon* Ait) is a member of the heath family native to North America. The first cultivation occurred around 1816 in sandy 'bogs', marshes or rain-soaked salt meadows in Massachusetts, New Jersey, Wisconsin, Washington and Oregon (Sialiano, 1998). The fruit is widely used in a variety of food products including juices and sauces. There is considerable evidence to support the use of cranberries for the prevention of urinary tract infections (Bodel et al., 1959; Moen, 1962; Sternlieb, 1963; Papas et al., 1968; Avorn et al., 1994). This effect is achieved by inhibiting the infecting bacteria, *Escherichia coli*, from adhering to uroepithelial cells (Sobota, 1984; Schmidt and Sobota, 1988; Zafriri et al., 1989; Ofek et al., 1991). Bacterial adherence to mucosal cells is a critical step in the development of infection (Beachey, 1981). It is hypothesized that cranberry juice acts by preventing adhesion which presumably facilitates urinary flushing of the causative bacteria, preventing their colonization of the urinary tract.

Adherence is facilitated by *E. coli* fimbriae which are proteinaceous fibers on the bacterial cell wall. Fimbriae produce adhesins that attach to specific carbohydrate receptors on uroepithelial cells (Beachey, 1981). Fructose, common in most fruits including cranberry, has been implicated in inhibiting adherence of *E. coli* phenotypes possessing type 1 (mannose-sensitive) fimbrial adhesins (Zafriri et al., 1989). A specific oligosaccharide receptor α-Gal(1 → 4)β-Gal has been shown to inhibit adherence of *E. coli* with P-type (mannose-resistant) fimbrial adhesins (Beachey, 1981). Recently, proanthocyanidin extracts were isolated from cranberries by bioassay-directed fractionation and exhibited antiadherence activity against uropatho-

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genic P-fimbriated *E. coli* (Howell et al., 1998). This report concerns the structural elucidation of the bioactive proanthocyanidins from the ethyl acetate extract of cranberry fruit.

2. Results and discussion

The proanthocyanidin fraction isolated from the ethyl acetate extract was tested for the ability to prevent adherence of *E. coli* to mannose-resistant adhesin by measuring the ability to prevent agglutination of both isolated P-receptor resin-coated beads and human erythrocytes. The proanthocyanidin fraction exhibited potent antiadherence activity at a concentration as low as 75 μg/ml, suggesting proanthocyanidins from the ethyl acetate extract of cranberry fruit could be beneficial in the prevention of urinary tract infections caused by P-fimbriated *E. coli*.

Confirmation of the proanthocyanidin constitution of the ethyl acetate fraction was made by heating with 5% HCl in *tert*-butanol which yielded cyanidin as the principal pigment. The dominance of the procyanidin unit of the polymeric sample was corroborated by ^{13}C NMR (Fig. 1) which showed strong peaks at 115–116 ppm and 119.8–121.1 ppm consistent with the C2′, C5′ and C6′ chemical shifts, respectively, of the catechol B-ring, with the almost total exclusion of carbon chemical shifts which may be attributed to the B-ring of other hydroxylation pattern. While most of the remaining carbon chemical shifts were consistent with a flavan-3-ol backbone, additional signals were identified suggesting that the polymers were different from most commonly encountered procyanidins (Foo and Porter, 1981; Porter et al., 1982; Foo et al., 1982). The most apparent observation is the presence of additional carbon signals at 151–152 ppm and a quaternary carbon peak at 104.3 ppm. Similar to the ^{13}C NMR spectrum of the doubly linked procyanidin dimer A2 (**1**) (isolated from the ethanol washings of the Sephadex LH20 column), the spectra of the polymers exhibited chemical shifts in the region consistent with C5 and C7 of the phloroglucinol A-ring involved in the double linkage. The chemical shift of the ketal carbon (C2) formed as a result of this additional bond observed at 104.7 ppm in **1** provided further support for A-type linkage. Another diagnostic feature of a doubly linked procyanidin is the pronounced upfield shift position of the methine C4 (δ 29–30) involved in interflavanoid bond (Nonaka et al., 1983; Balde et al., 1991) but the significance of this signal was obscured by the unsubstituted C4 methylene carbon resonances of the terminating flavanol unit which also occurred in this region. This complication was overcome by the

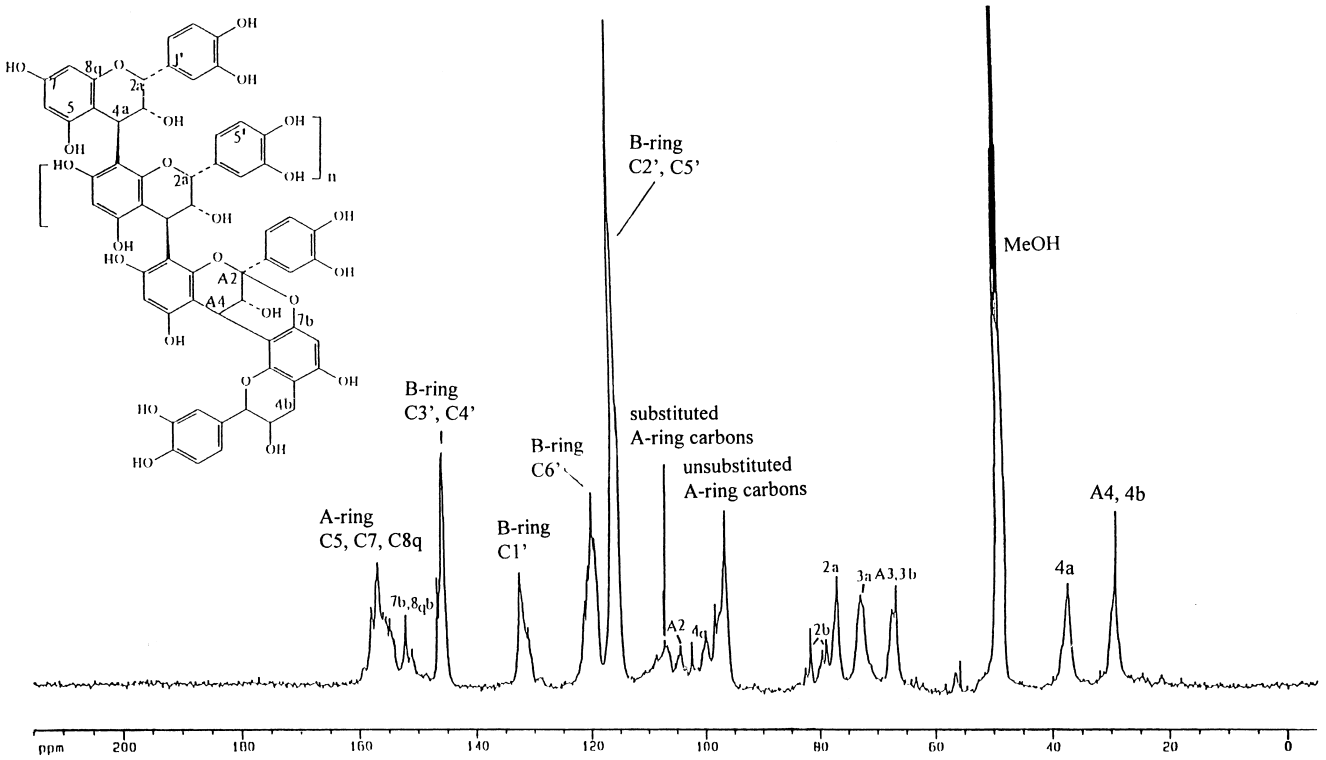


Fig. 1. ^{13}C NMR spectrum of cranberry proanthocyanidins showing the presence of A-type linkages.

use of DEPT which showed the presence of both the methine and methylene carbons in the carbon signals at 29.4 ppm of the polymer. Further confirmation was also made by using H–C correlation studies (HSQC) which showed that the carbon peak (δ 29.4) was correlated to both the H4 methylene protons at δ 2.6–3.2 of the terminating flavanol and the aryl substituted lower field H4 methine protons at δ 4.2. Other changes including the downfield shift of the 2,3-*cis* C2 resonances to the corresponding 2,3-*trans* carbon position and the upfield shift of the extender C3 resonances to the terminal C3 region associated with the doubly linked flavanol units caused difficulties in assessing the nature of the stereochemistry of the constitutive units and in the estimation of the number average molecular weight of the polymer using ^{13}C NMR data.

To obtain more detailed information on the chemical structures of the cranberry proanthocyanidins, the polymer was subjected to partial cleavage with dilute acid in the presence of excess phloroglucinol. Chromatography of the reaction products over Sephadex LH20 yielded epicatechin (**2**), catechin-(4α-2)-phloroglucinol (**3**), epicatechin-(4β-2)-phloroglucinol (**4**), epigallocatechin-(4β-2)-phloroglucinol (**5**), epicatechin-(4β-8)-epicatechin-(4β-2)-phloroglucinol (**6**), and epicatechin-(4β-8, 2β-*O*-7)-epicatechin or A2 (**1**). The identities of all these reaction products were confirmed by chromatographic and spectroscopic comparison with authentic samples (Foo et al., 1996). A small amount of an impure fraction was also isolated, which on subjecting to electrospray mass spectral analysis gave a strong $[M - H]^-$ peak at m/z 699 which was consistent with the elemental composition of epicatechin-(4β-8, 2β-*O*-7)-epicatechin-(4β-2)-phloroglucinol (**7**). However, the identity of **7** in the absence of NMR data due to the small quantity and low quality of the sample had to be regarded as tentative only. The relative high yield of the various epicatechin phloroglucinol adducts (Table 1) compared to other phloroglucinol adducts indicated that the cranberry proanthocyanidin oligomers were made up of predominantly epicatechin extender units which were represented by the phloroglucinol adducts **4** and **6**. The isolation of a small amount of **5** indicated the presence of a low level (< 5%) of prodelphinidin unit which was not apparent from the ^{13}C NMR spectrum. As was also apparent from the degradation products, the stereochemistry of the constitutive flavanol units was predominantly of the 2,3-*cis* type but a small proportion of the 2,3-*trans* units was also present as indicated by the isolation of catechin-(4α-2)-phloroglucinol (**3**). While the determination of the ratio of the 2,3-*cis* to 2,3-*trans* stereochemistries could in most instances be readily achieved with ^{13}C NMR by virtue of the distinct differences in their respective C2 chemical shifts (Czochanska et al., 1980), the existence of doubly linked procyanidin units where the C2 of the lower flavanol had shifted downfield to the 2,3-*trans* general region as for example at 82.2 ppm in A2, had created uncertainty with this estimation. In this instance, the degradation results clearly supported that the carbon resonances at 81.5–82.5 ppm observed in the ^{13}C NMR spectrum (Fig. 1) of the purified cranberry polymer were contributed mostly by the C2 of the doubly linked flavanol units. The phloroglucinol degradation reaction in addition to defining the extender units in the form of phloroglucinol adducts also provided information on the type of terminating units in the form of unsubstituted flavanols. Epicatechin and A2 in a ratio of about 1–4, respectively, were the two such compounds isolated from the reaction mixture indicating that the procyanidin polymer was more frequently terminated with A2 and to a lesser extent with epicatechin. The reaction products that presumably retained the original interflavanoid bonds were **1** and **6** suggesting that the C4/C8 was the most common form of interflavanoid linkage.

Table 1
Yield of isolated degradation products from reaction of the cranberry proanthocyanidins with phloroglucinol

Product	Yield (mg)
Epicatechin (**2**)	15
Catechin-(4α-2)-phloroglucinol (**3**)	12
Epicatechin-(4β-2)-phloroglucinol (**4**)	108
Epigallocatechin-(4β-2)-phloroglucinol (**5**)	7
Epicatechin-(4β-8)-epicatechin-(4β-2)-phloroglucinol (**6**)	20
Epicatechin-(4β-8, 2β-*O*-7)-epicatechin or A2 (**1**)	60
Epicatechin-(4β-8, 2β-*O*-7)-epicatechin-(4β-2)-phloroglucinol (**7**)	15

The remaining outstanding question that needed to be resolved was related to the molecular size of the cranberry proanthocyanidin sample. The validity of taking advantage of the signal intensity of the well-separated C3 signals of the terminating flavanol unit (δ 67–68) and the extending unit (δ 72–73) to obtain number average molecular weight from the ^{13}C NMR spectrum had been demonstrated (Czochanska et al., 1980). The application of this simple technique for estimating molecular weight, however, was complicated by the presence of doubly linked procyanidin unit. As observed in the ^{13}C NMR spectrum of A2, there was a pronounced downfield shift of the upper C3 to 68.5 ppm which was close to the region (67.4 ppm) of the corresponding C3 of the terminating flavanol. Such changes caused an exaggerated effect as the peak intensity at the 67–68 ppm region where the terminating C3 carbon signal occurred was increased at the expense of the signals at the 72–73 ppm region giving the appearance that the molecular weight was much lower than expected. This anomaly was apparently in the ^{13}C

NMR of the cranberry proanthocyanidin sample where the ratio of the area of the carbon signals at 67–68 ppm and 72.8 ppm was about 1.0:1.3, respectively. To overcome this problem mass spectra using electrospray (ES-MS) and matrix-assisted laser absorption time-of-flight (MALDI-TOF) mass spectrometry were obtained on the proanthocyanidin sample.

The ES-MS spectrum (Fig. 2) using negative ion detection showed molecular ion species consistent with procyanidin oligomers containing a doubly linked unit. The observation of peaks at *m/z* 1439.3 and 1151.6 were consistent with a procyanidin pentamer (DP 5) and tetramer (DP 4), respectively with each containing a doubly linked unit. A small ion peak at *m/z* 1725.0 suggested a small amount of a hexamer (DP 6) with a doubly linked unit present in the chain. Other ion peaks with the most intense signals at *m/z* 575.3, 719.4 and 862.6 were also present. These were due to doubly charged ions as were apparent from their narrower signal width compared to the singly charged species. Such multiply charged species were more frequently observed in ES-MS (Foo and Lu, 1999) and became more intense at the expense of singly charged species as the molecular weight increased, probably as a result of longer chain length which allowed for better charge separation, thus minimizing the electrostatic repulsive forces. The ion peaks at *m/z* 575.3 and 719.4 were attributed to the doubly charged species of the tetra-

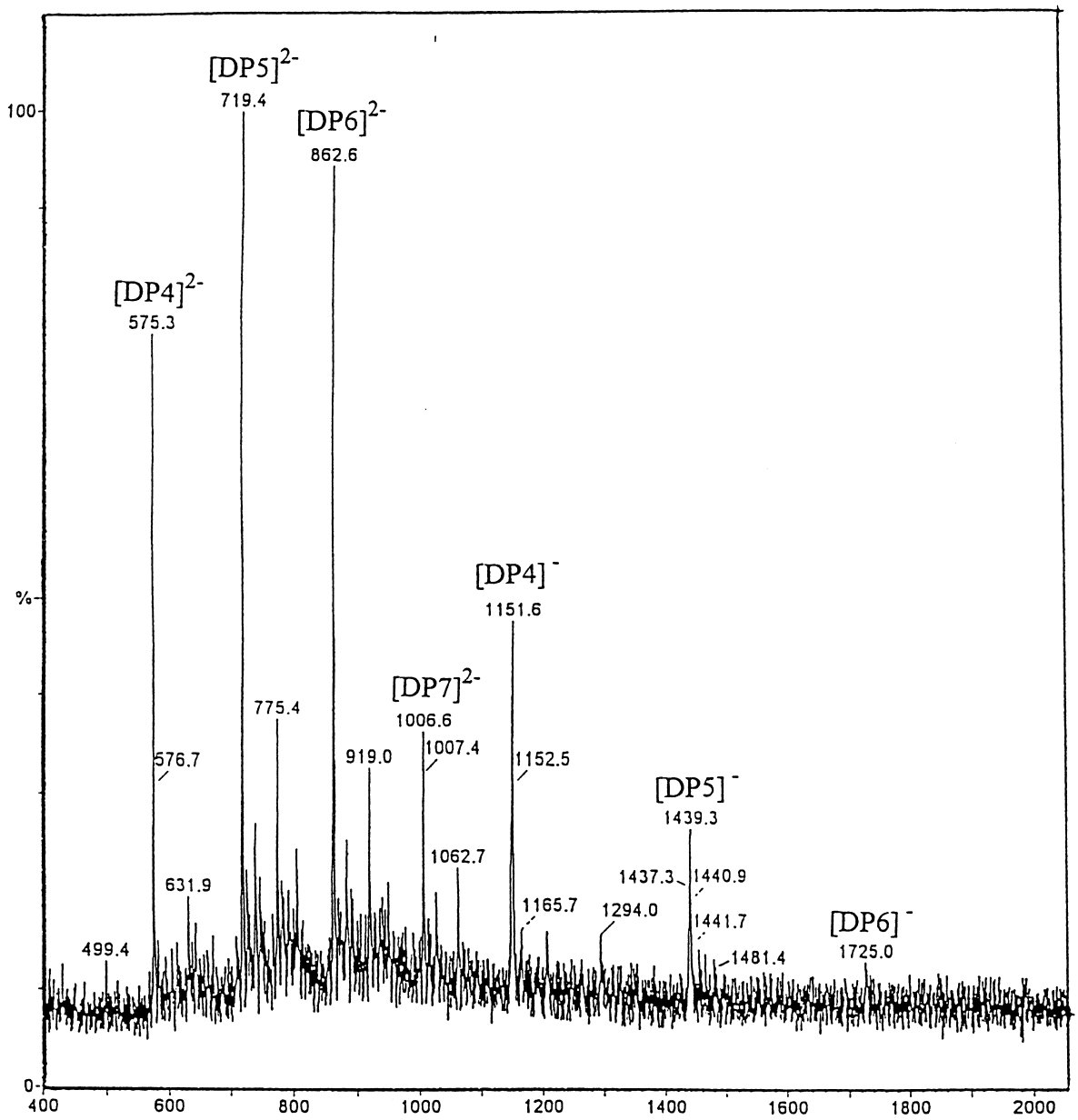


Fig. 2. Electrospray mass spectrum of cranberry proanthocyanidins in the negative-ion mode.

meric and pentameric procyanidins which were represented by the singly charged signals at m/z 1151.6 and 1439.3, respectively. The tetramers and pentamers, therefore, were the dominant entities in the cranberry proanthocyanidin sample although a small amount of the higher molecular weight species were also detected as indicated by the observation of a weak ion peak at m/z 1725.0 which corresponded to a procyanidin hexamer containing an A-type linkage and its corresponding but much more intense doubly charged ion at m/z 862.6. In addition, the observation of a relatively small doubly charged peak at m/z 1007.4 suggested that a heptamer with an A-type linkage was also present in the sample although the corresponding singly charged ion was not observed in the ES-MS spectrum.

The MALDI-TOF mass spectrum (Fig. 3) of the cranberry proanthocyanidins recorded as sodium ion adducts in the positive ion mode showed up two principal ion peaks at m/z 1173 and 1464 which were consistent with the sodium ion adducts of the procyanidin tetramer (DP 4) and pentamer (DP 5), respectively each containing an A-type linkage. Higher molecular weight ions but with significantly less signal intensity were also observed at m/z 1751 and 2038 which were attributed to the procyanidin hexamer (DP 6) and heptamer (DP 7), respectively, with each possessing an A-type linkage. These observations fully corroborated the interpretation accorded to the ES-MS data and demonstrated that both these mass spectral techniques were comparable in usefulness for the analysis of low to moderate size proanthocyanidin polymers. Suggestion had been made that because the MALDI-TOF method did not cause fragmentation of the oligomers, the observed peak distribution, therefore, reflected the quantity of the various oligomers present in the

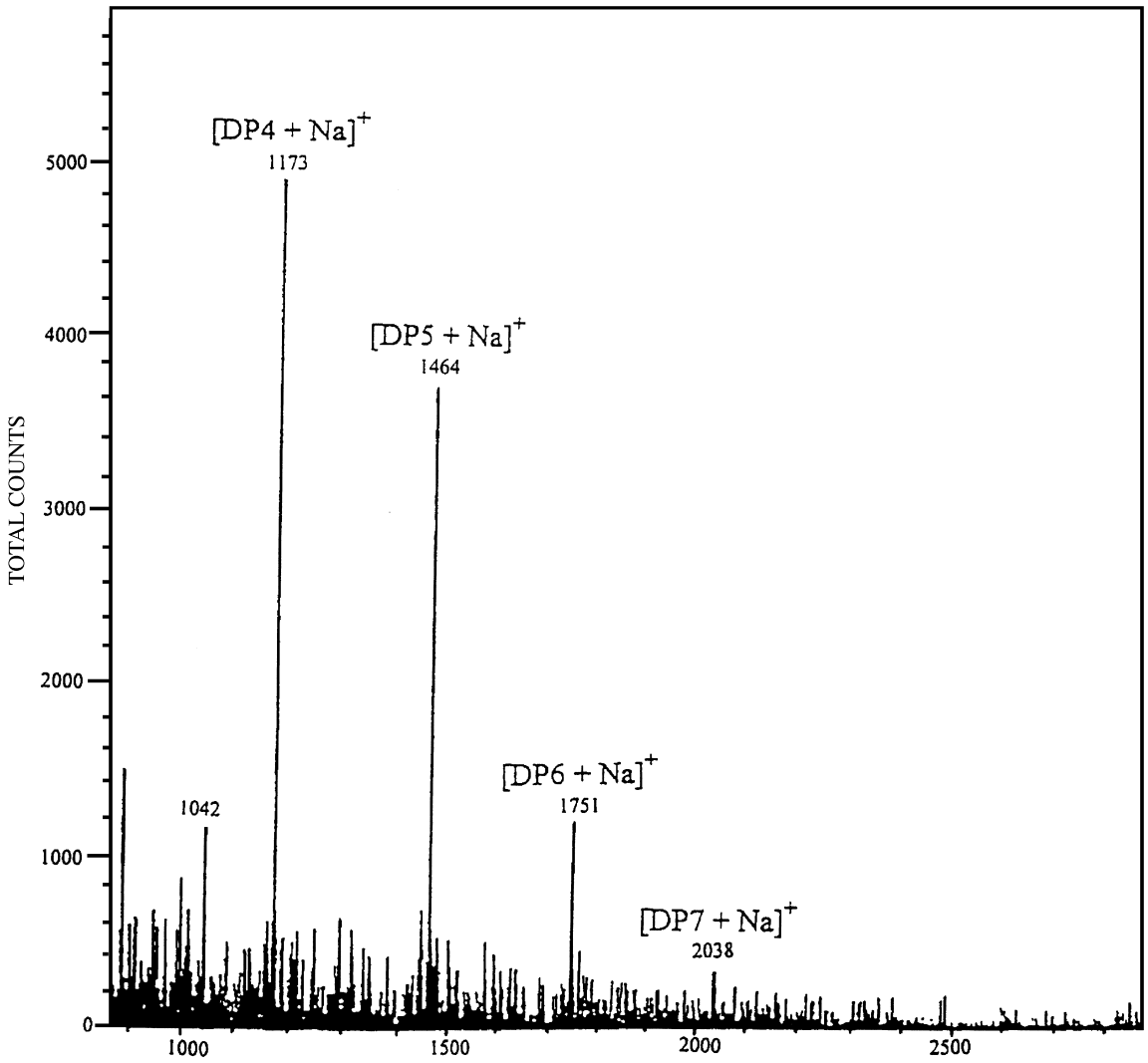


Fig. 3. MALDI-TOF mass spectrum of cranberry proanthocyanidins in the positive-ion mode.

sample (Ohnishi-Kameyama et al., 1997). By using signal height as a measure of relative abundance, the contributions of the various procyanidin oligomers in the MALDI-TOF mass spectrum of the cranberry sample were 48.6% for DP 4, 36.6% for DP 5, 11.6% for DP 6 and 3.1% for DP 7. Calculation based on these distributions gave a number average molecular weight of 1354 or an average of 4.7 flavanol units for the cranberry sample.

The validity of such estimation could be checked against the ^{13}C NMR data. It was noted earlier that the areas of the C3 resonances for the extender and terminal flavanol could be integrated to obtain the number average molecular weight. However, the application of this technique was complicated by the presence of A-type linked unit which caused a large upfield shift of the C3 resonance of the pyran ring involved in the double linkage to the region observed for the terminal C3 resonances. From the phloroglucinol reaction products and the mass-spectral data it could now be reasonably assumed that there was on average an A-type unit present. Thus, if the number average molecular weight of the procyanidin was about 4.7 flavanol units as derived from MALDI-TOF mass spectrum, there would be on average 3.7 extenders to 1 terminal flavanol.

To take into account the anomaly in the ^{13}C NMR spectrum caused by the presence of an A-type unit in the proanthocyanidin sample, the signal size at 67–68 ppm would be expected to increase by an average of one carbon to 2.0 as a result of the contribution of the highfield position of the C3 resonance and the signal at 72–73 ppm decreased by the corresponding number to an average of 2.7 carbon units. The ratio of the two calculated magnitude of the carbon signals was 1:1.35 which was comparable to the value 1:1.3 obtained by direct integration of the actual carbon signals from the ^{13}C NMR spectrum. Such consistent results provided further confirmation that the ethyl acetate extracted cranberry proanthocyanidin sample consisted of predominantly procyanidin tetramers and pentamers with 2,3-*cis* stereochemistry linked mostly via C4/C8 interflavanoid linkages and terminated mostly by A2 as shown in structure **8**. Other structural variations were also present such as a small proportion of catechin and epigallocatechin extender units. A small proportion of the proanthocyanidins had an epicatechin as the terminating unit and these on average would have an A2 as part of the extenders to account for the observed mass ion peaks and the tentative identification of the corresponding A2 phloroglucinol adduct **7**. These finer structural features of the proanthocyanidins were not apparent from the ^{13}C NMR and mass spectral data and were only revealed by analysis of the degradation products with phloroglucinol.

3. Experimental

NMR spectra were recorded on a Bruker Avance 300 NMR spectrometer operating at 300.13 MHz for ^{1}H and 75.45 MHz for ^{13}C in CD_3OD. Mass spectra were obtained from a VG Plateform Electrospray mass spectrometer performed in the negative ion detection mode and from a PHI-EVANS MALDI triple electrostatic analyser time-of-flight mass spectrometer in the positive ion detection mode using 2,5-dihydroxybenzoic acid as the matrix. TLC was performed on Schleicher and Schnell cellulose plates developed with HOAc–H_2O (3:47) and visualized with vanillin–HCl spray.

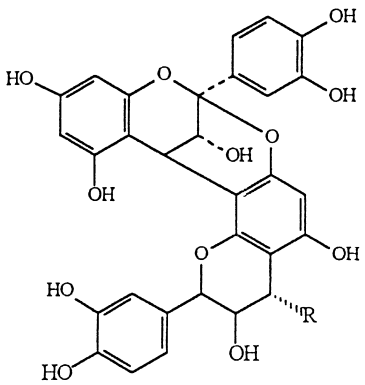
3.1. Extraction and isolation of proanthocyanidins

The washed cranberry fruit (10 kg) was homogenized with acetone in a blender for 10 min at 4°C. The resulting mixture was filtered and the pulp discarded. The clarified extract was concentrated under reduced pressure to remove acetone, the residue diluted with H_2O and defatted by extracting three times with an equal volume of petroleum ether. The aqueous phase was reduced to a small volume and extracted four times with an equal volume of EtOAc. The EtOAc extracts were combined and concentrated and the resulting residue freeze-dried to yield a red-colored syrup (47 g). The syrup (45 g) was dissolved in EtOH and applied onto a Sephadex LH20 column. The column was washed with EtOH until the eluant was clear. The adsorbed proanthocyanidins were eluted from the column with 60% aqueous acetone. The acetone was removed on a rotary evaporator at about 40°C and the resulting residue freeze-dried to give a light brown powder (1.5 g).

3.2. Bioactivity testing

Strains Standard methods for detection of antiadherence bioactivity require utilizing a surface that contains the putative disaccharide α-Gal(1 → 4)β-Gal receptor that is found on the surface of uroepithelial cells (Kallenius et al., 1982). This Gal–Gal sequence is similar in structure to the P blood group antigen on the surface of human red blood cells (HRBC) (Kallenius et al., 1980). Tests used to detect antiadherence bioactivity included measuring ability of the proanthocyanidin extract to suppress agglutination of either HRBCs (A_1, Rh+) (Evans et al., 1977) or latex beads coated with synthetic P receptor analog (De Man et al., 1987) following incubation with P-fimbriated *E. coli*.

Uropathogenic *E. coli* strains were isolated from the urine of human patients suffering from urinary tract infections. Strains specific for P fimbrial adhesins were subcultured in tryptose broth at 37°C for 16 h and



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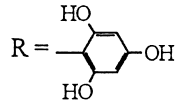
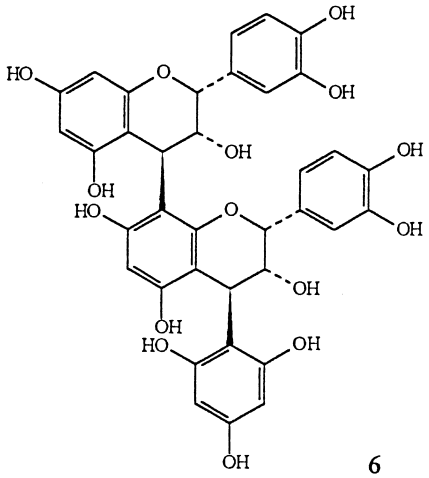
7 R = 



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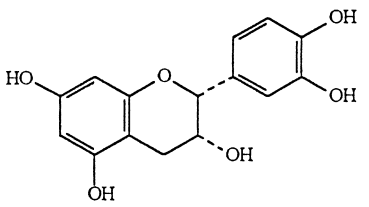

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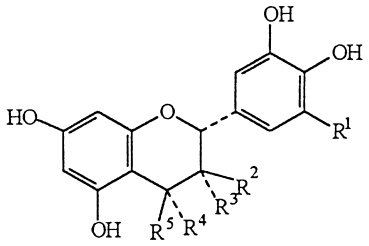

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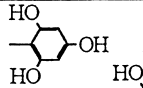
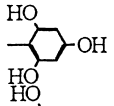
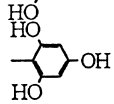
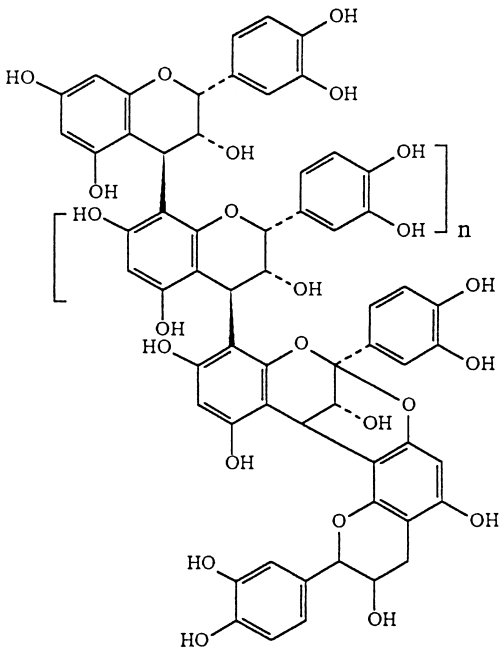
3	H	OH	H		H
4	H	H	OH	H	
5	OH	H	OH	H	



8 where n = 1 or 2

transferred to colonization factor agar (CFA) plates (Evans et al., 1977) and grown overnight at 37°C to enhance production of P fimbriae. Strains were harvested by centrifugation, washed once and suspended in phosphate buffered saline solution (PBS) at pH 7.0 at a concentration of 5×10^8 bacteria/ml. Proanthocyanidin extract (1.2 mg) was dissolved in 1 ml of PBS and neutralized with 1 N NaOH. Serial two-fold dilutions were prepared. A 30-μl drop of each dilution was incubated with 10 μl of bacterial suspension on a 24-well polystyrene plate for 10 min at room temperature on a rotary shaker. Freshly drawn HRBCs (A_1, Rh+) and P-receptor coated beads were each suspended (3%) in PBS and added separately (10-μl drops) to test suspensions. Suspensions were incubated for 20 min on a rotary shaker at room temperature and evaluated microscopically for the ability to prevent agglutination. The final dilution was recorded at which agglutination suppression by the proanthocyanidins fraction occurred. Controls included wells containing

bacteria + PBS, HRBC or P-beads + PBS, bacteria + test fraction, HRBC or P-beads + test fraction, and bacteria + HRBC or P-beads.

3.3. Reaction with phloroglucinol

To a mixture of the proanthocyanidin (900 mg) and phloroglucinol (700 mg) was added 1% HCl in EtOH (5.0 ml) and the resulting mixture was shaken until complete solution was obtained. After 24 h standing at ambient temperatures the reaction mixture was diluted with H_2O (5.0 ml) and the resulting solution applied onto a column of Sephadex LH20 prepared with 50% aqueous EtOH. The column was eluted with 50% aqueous EtOH and fractions (18 ml) were collected and monitored by TLC.

3.4. Proanthocyanidin oligomers (**8**)

$[\alpha]_D + 105^\circ$ (*c* 0.10 MeOH); UV (MeOH) λ_{max} 280 nm; ES-MS (see Fig. 2); MALDI-TOF MS (see Fig. 3) and ^{13}C NMR (see Fig. 1). ^1H NMR (CD_3OD): δ 2.6–3.3 (H4 of terminal units), 3.7–4.5 (H3 of extending units), 3.9–4.5 (H3 of terminating units), 4.1–4.8 (H4 of A-type unit), 4.4–5.0 (H4 of extending units), 4.4–5.8 (H2 of both extending and terminating units), 5.7–6.3 (H6, H8 of phloroglucinol A-ring), 6.3–7.6 (H2′, H5′ and H6′ of B-ring).

3.5. Epicatechin (**2**)

R_f 0.32. ^{13}C NMR (CD_3OD): δ 29.1 (C4), 67.4 (C3), 79.7 (C2), 95.8 (C8), 96.2 (C6), 100.1 (C4a), 115.3 (C2′), 115.7 (C5′), 119.4 (C6′), 132.2 (C1′), 145.7, 145.9 (C3′, C4′), 157.4, 157.6, 157.9 (C5, C7, C8a). ^1H NMR (CD_3OD): δ 2.7–2.86 (*m*, H4), 4.21 (*m*, H-3), 4.85 (*s*, H2), 5.94 (*d*, J = 2.2 Hz, H6), 6.02 (*d*, J = 2.2 Hz, H8), 6.81 (*m*, H5′, H6′), 7.04 (*bs*, H2′).

3.6. Epicatechin-(4β-8, 2β-O-7)-epicatechin or A2 (**1**)

R_f 0.28; $[\alpha]_D + 24^\circ$ (*c* 0.15, MeOH). ^{13}C NMR (CD_3OD): δ 29.7 (C4 upper), 30.3 (C4 lower), 67.4 (C3 lower), 68.5 (C3 upper), 82.2 (C2 lower), 98.8 (C6 upper), 97.1 (C6 lower), 100.8 (C4a lower), 102.9 (C4a upper), 107.7 (C8 lower), 116.1–116.5 (C2′, C5′ upper and lower), 120.2 (C6′ upper), 120.9 (C6′ lower), 131.6 (C1′ lower), 132.9 (C1′ upper), 146.0–147.1 (C3′, C4′ upper and lower), 152.8 (C8a lower), 152.7 (C7 lower), 154.7 (C8a upper), 156.9–158.5 (C5 lower, C5, C7 upper). ^1H NMR (CD_3OD): δ 2.5–2.9 (*m*, 2 × H4 lower), 3.97 (*d*, J = 3.5 Hz, H3 upper), 4.14 (*bs*, H3 lower), 4.32 (*d*, J = 3.5 Hz, H4 upper), 4.82 (*s*, H2 lower), 5.95 (*d*, J = 2.3 Hz, H8 upper), 5.98 (*d*, J = 2.3 Hz, H6 upper), 6.00 (*s*, H6 lower), 6.6–7.06 (H2′, H5′, H6′ upper and lower).

3.7. Catechin-(4α-2)-phloroglucinol (**3**)

R_f 0.62. ^{13}C NMR (CD_3OD): δ 38.9, 73.8, 84.9, 96.8, 97.6, 107.2, 115.7, 116.6, 121.1, 132.0, 147.1 (2×), 157.7–158.9. ^1H NMR (CD_3OD): δ 4.4–4.53 (*m*, 3H), 5.8–6.0 (*m*, 4H) and 6.4–7.2 (*m*, 3H).

3.8. Epicatechin-(4β-2)-phloroglucinol (**4**)

R_f 0.60. ^{13}C NMR (CD_3OD): δ 37.6, 73.6, 77.8, 95.9, 96.3, 97.1, 102.1, 107.4, 115.2, 116.0, 119.3, 132.7, 145.5, 145.8, 156.8, 157.8, 158.3. ^1H NMR (CD_3OD): δ 3.96, (*s*, 1H), 4.52 (*s*, 1H), 5.05 (*s*, 1H), 5.86 (*bs*, 2H), 5.96 (*s*, 2H), 6.73 (*m*, 2H), 6.90 (*s*, 1H).

3.9. Epigallocatechin-(4β-2)-phloroglucinol (**5**)

R_f 0.52. ^{13}C NMR (CD_3OD): δ 37.6, 73.6, 77.8, 95.9, 96.5, 97.1, 102.1, 107.4, 108.0, 132.0, 133.4, 146.6, 157–158. ^1H NMR (CD_3OD): δ 3.96 (*s*, 1H), 4.52 (*s*, 1H), 5.05 (*s*, 1H), 5.86 (*bs*, 2H), 6.01 (*s*, 2H) and 6.43 (*s*, 2H).

3.10. Epicatechin-(4β-8)-epicatechin-(4β-2)-phloroglucinol (**6**)

R_f 0.53. ^{13}C NMR (CD_3OD): δ 37.1, 37.2, 72.3, 73.3, 76.8, 76.9, 96–97, 99.8, 101.9, 107.0, 108.0, 115.1, 115.3, 115.7, 115.8, 132.5, 145.2, 145.3, 145.4, 145.6, 154.8, 157–158. ^1H NMR (CD_3OD): δ 4.02 (*bs*, 1H), 4.06 (*bs*, 1H), 4.62 (*bs*, 1H), 4.72 (*bs*, 1H), 5.08 (*bs*, 1H), 5.23 (*bs*, 1H), 5.62–5.85 (*m*, 5H), 6.7–7.2 (*m*, 6H).

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