A-Type Proanthocyanidin Trimers from Cranberry that Inhibit Adherence of Uropathogenic P-Fimbriated *Escherichia coli*

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Three proanthocyanidin trimers possessing A-type interflavanoid linkages, epicatechin-(4β→6)-epicatechin-(4β→8, 2β→*O*→7)-epicatechin (**4**), epicatechin-(4β→8, 2β→*O*→7)-epicatechin-(4β→8)-epicatechin (**5**), and epicatechin-(4β→8)-epicatechin-(4β→8, 2β→*O*→7)-epicatechin (**6**), were isolated from the ripe fruits of *Vaccinium macrocarpon* (cranberry) and prevented adherence of P-fimbriated *Escherichia coli* isolates from the urinary tract to cellular surfaces containing α-Gal(1→4)β-Gal receptor sequences similar to those on uroepithelial cells. The structure of **4** was elucidated by a combination of spectroscopic methods and acid-catalyzed degradation with phloroglucinol. Also isolated were the weakly active epicatechin-(4β→8, 2β→*O*→7)-epicatechin (procyanidin A2) (**3**) and the inactive monomer epicatechin (**1**) and the inactive dimer epicatechin-(4β→8)-epicatechin (procyanidin B2) (**2**).

Cranberry (*Vaccinium macrocarpon* Ait., family Ericaceae) fruit juice has been used traditionally for the treatment and prevention of urinary tract infections, although it was only recently that its effectiveness was scientifically demonstrated by a randomized, double-blind placebo-controlled trial.[1] High levels of benzoic acid have been reported in cranberry juice,[2] and a possible mechanism for the bacteriostatic effect of cranberry juice was suggested to be due to acidification of the urine.[3,4] Recent studies, however, have indicated a different mechanism involving the inhibition of certain types of bacterial adherence to the epithelial cells that line the urinary tract, thus preventing bacterial colonization and subsequent infection.[5,6]

The attachment of *Escherichia coli*, the principal bacterial species responsible for urinary tract infection, is facilitated by fimbriae, which are proteinaceous fibers on the bacterial cell wall. Fimbriae produce specific adhesins that attach to specific oligosaccharide receptors on uroepithelial cells.[7] Two components in cranberry juice have been shown to inhibit the adherence of *E. coli* to uroepithelial cells. The first is fructose, which is common to many fruit juices and has been implicated in inhibiting adherence of *E. coli* phenotypes possessing type 1 (mannose-sensitive) fimbriae.[8] The second was recently demonstrated to be the proanthocyanidins, which are responsible for inhibiting adherence of the P-fimbriated *E. coli* (mannose-resistant) fimbriae, although specific individual compounds were not structurally defined in that study.[9]

The present investigation was undertaken to elucidate the chemical structures of the lowest molecular weight cranberry fruit proanthocyanidins that exhibit in vitro anti-adherence activity of P-fimbriated *E. coli*.

Results and Discussion

Column chromatography of the ethyl acetate extract of the fruit of *V. macrocarpon* alternating between Sephadex LH-20 and MCI Gel CHP 20P yielded epicatechin (**1**) and five proanthocyanidins (**2**−**6**) (See Chart 1). Compound **2** was identified as procyanidin B2 or epicatechin-(4β→8)-epicatechin from its negative-ion ESIMS (*m*/*z* 577) and ^{13}C NMR data.[10] Compound **3** gave a molecular ion at *m*/*z* 575 with ESIMS operating in the negative-ion mode and, in being two mass units less than procyanidin B2, provided initial evidence that it was a doubly linked procyanidin dimer of the A-type. This deduction was supported by its distinctive ^{13}C NMR spectrum, which confirmed **3** to be procyanidin A2 (epicatechin-(4β→8, 2β→*O*→7)-epicatechin).[11,12]

The ESIMS of compound **4** gave an $[M - H]^-$ peak at *m*/*z* 863, which was consistent with a trimeric procyanidin constitution in which an A-type linkage was also present. This was also evident from its ^{13}C NMR spectrum, which showed in addition to the expected more common procyanidin B-type chemical shifts[13] the presence of signals at 30.0, 68.2, and 104.9 ppm, which were diagnostic of the respective C-4, C-3, and the quaternary C-2 chemical shifts of the pyran ring directly involved in the A-type interflavanoid bonding,[11,12] as earlier outlined. On acid-catalyzed degradation with phloroglucinol, compound **4** yielded epicatechin-(4β→2)-phloroglucinol, which was identified by HPLC comparison with authentic material,[14] indicating that the trimer consisted of an epicatechin extender unit which was linked to the A2 unit via a 4β→6 or 4β→8 interflavanoid bond. The specific position of this linkage was established by examination of the long-range proton−carbon couplings (HMBC) of **4**. In this connection attention was focused on the unsubstituted aromatic proton (δ 6.08), which from the HSQC and HMBC data had been identified as belonging to the phloroglucinol A-ring of the doubly linked flavan moiety involved in the interflavanoid bonding with the epicatechin extender unit. This proton was observed to be long-range coupled to the substituted carbon (108.9 ppm) as well as the ketal carbon (104.9 ppm). Hence the proton at δ 6.08 was that of H-8 and the substituted A-ring carbon at 108.9 ppm had to be the C-6, establishing the position of interflavanoid linkage to be on this carbon. Compound **4** was therefore determined structurally as epicatechin-(4β→6)-epicatechin-(4β→8, 2β→*O*→7)-epicatechin.

The ESIMS of compounds **5** and **6** also gave a single $[M - H]^-$ peak at *m*/*z* 863, which suggested they both have a

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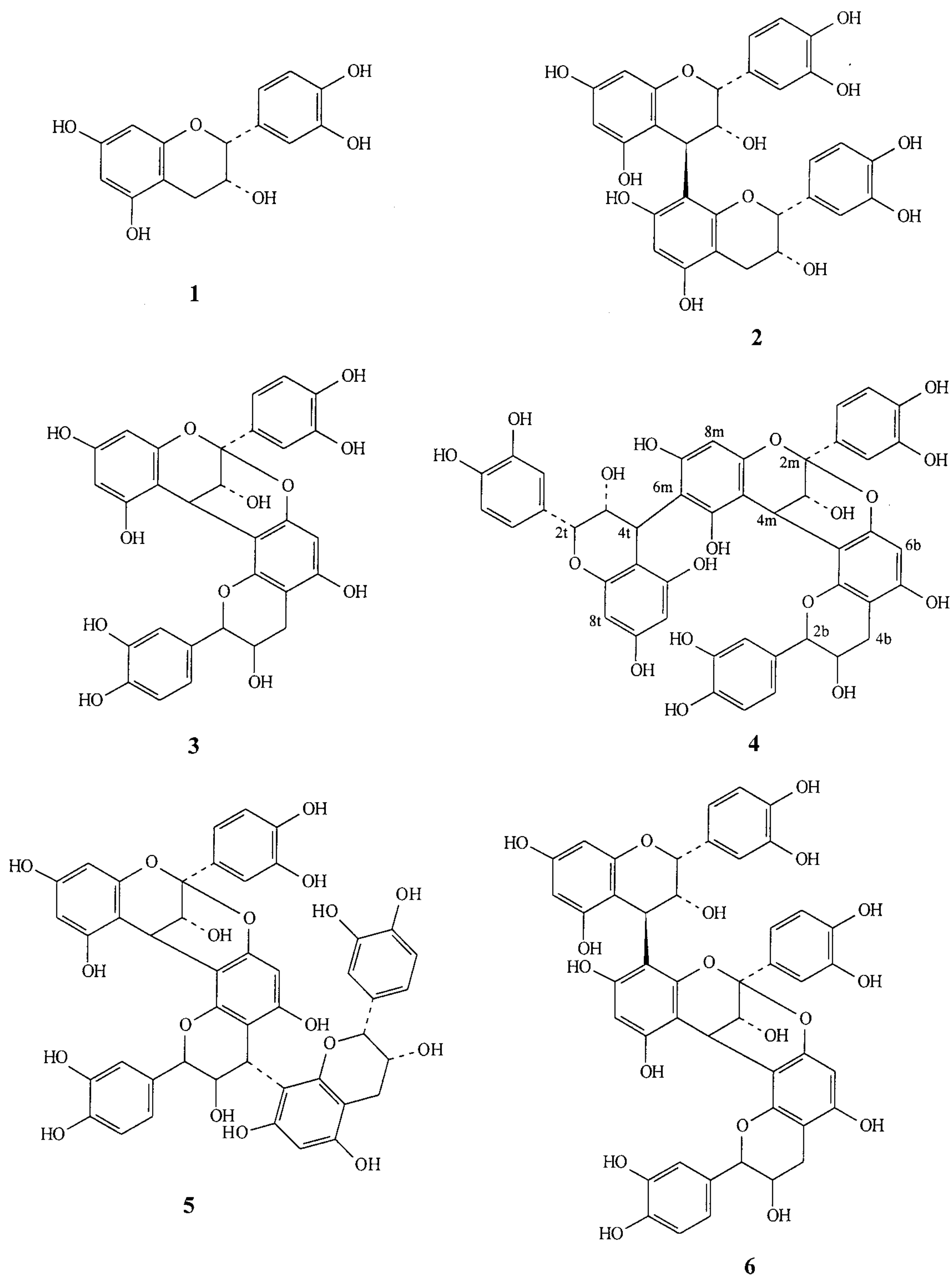
Chart 1



trimeric procyanidin constitution similar to **4**. Unlike the relatively uncomplicated ^{13}C NMR spectrum of **4**, the spectrum of **5** contained more carbon signals of different intensities than those that could be accounted for in a procyanidin trimer. HPLC analysis showed the sample to be predominantly a single compound, suggesting that the multiple carbon signals observed were likely a result of conformational isomerism due to steric hindrance to rotation about the interflavanoid bond.[15] Such a phenomenon was particularly common among proanthocyanidins containing the rigid A-type units in the chain which give rise to multiple NMR signals of different intensities.[15,16] The observation of the corresponding carbon chemical shifts at 80.6 and 79.2 ppm for C-2 carbons, 72.9 and 67.5 ppm for the C-3 carbons, and 37.6 and 28.2 ppm for the flavanyl-substituted and -unsubstituted, respectively, benzylic C-4 carbons of the two epicatechin moieties further indicated the presence of an epicatechin unit that was not tied up in the A-unit. This served as the bottom or terminating unit of the trimer **5**. The location of the interflavanoid linkage on the epicatechin terminating unit was deduced from the proton NMR data, which showed that H-2 of this unit (assignment confirmed by HMQC) was at an unusually downfield position at δ 5.75 due to the proton being in close proximity to the upper A-ring in the deshielding region. A Dreiding model showed this can only occur if the interfla-

Table 1. ^{13}C NMR Data for Compound **4**[a]

carbon	top unit	middle unit	bottom unit
2	77.6	104.9	82.2
3	73.7	68.2	67.3
4	37.8	30.0	30.5
4a	100.9	100.0	102.9
5	158.7	155.9	157.5
6	97.0	108.9	97.0
7	158.3	158.3	152.7
8	97.0	96.6	109.2
8a	156.0	157.1	152.7
1′	133.0	131.6	132.8
2′	115.8	116.1	115.8
3′	146.3	147.2	146.7
4′	146.1	146.8	146.3
5′	116.1	116.4	116.9
6′	119.8	120.5	120.9

[a] Chemical shifts (ppm) are expressed relative to the CD_3OD peak at 49.5 ppm.

Table 2. Effects of Compounds **1**−**6** to Inhibit Adherence of Uropathogenic P-Fimbriated *E. coli*

	anti-adherence activity (mg/mL)[a]				
compound	2.4[b]	1.2	0.6	0.3	0.15
1	−	−	−	−	−
2	−	−	−	−	−
3	+	−	−	−	−
4	+	+	+	±	−
5	+	+	+	±	−
6	+	+	±	−	−

[a] Positive anti-adherence activity (+) is measured as the ability of the compounds to suppress agglutination of both human red blood cells (A_1, Rh+) and latex beads coated with synthetic P receptor analogue following incubation with P-fimbriated *E. coli.* [b] Dilution of compounds **1**−**6** in PBS, neutralized with 1 N NaOH.

vanoid bond is at C-8 of the A-ring of the epicatechin; therefore, **5** was assigned as epicatechin-(4β→8, 2β→*O*→7)-epicatechin-(4β→8)-epicatechin, a structure consistent with published data.[17,18]

The observation of the C-2 at higher field (77.5 ppm) in the ^{13}C NMR spectrum of **6**, characteristic of the upfield shift[13] as a result of an aryl substituent at C-4, suggested that the "unencumbered" epicatechin unit was the extender unit attached to the top end of the trimer. Compounds **4** and **6** were therefore isomeric, and since the former had been established from long-range coupling experiments to be the C-4/C-6 linked compound, **6** was concluded to be the C-4/C-8 linked regioisomer epicatechin-(4β→8)-epicatechin-(4β→8, 2β→*O*→7)-epicatechin. The validity of this structural assignment was also supported by the use of a Dreiding model, which showed that in the C-4/C-8 linked isomer there were steric interactions between both the A- and B-rings of the extender epicatechin unit and the lower B-ring. This restricted rotation about the single interflavanoid bond thus accounted for the observed conformational isomerism in **6**. A trimeric procyanidin of like constitution, aesculitanin A, has been described from the seeds of *Aesculus hippocastanum.*[15]

All three procyanidin trimers (**4**−**6**) possessed anti-adherence activities as determined by human erythrocyte and P-receptor resin-coated bead agglutination suppression when incubated with P-fimbriated *E. coli* (Table 2). Epicatechin **1** and its dimer **2** had no activity, while the A-linked dimer **3** demonstrated weak activity. Studies have shown that while *E. coli* with P-type fimbriation can cause both cystitis (lower urinary track infection) and pyelonephritis (upper urinary track infection), it may be more instrumental in the development of the latter.[19,20] Proanthocyanidin has been detected in the plasma[21] of rats fed orally with proanthocyanidin-rich extracts, and the presence in cranberry of proanthocyanidins such as the trimers described herein may contribute, at least in part, to the observed health benefits accorded to consumers of cranberry beverages.

Experimental Section

General Experimental Procedures. A Perkin-Elmer 241 polarimeter operating with a Na lamp was used for measuring optical rotations, and a Hewlett-Packard 8451 A diode array spectrophotometer was employed for UV measurement. A Bruker Avance 300 NMR spectrometer, operating at 300.13 MHz for ^1H and 75.47 MHz for ^{13}C, using the XWINNMR software package, was used for NMR experiments in CD_3OD. DEPT (distortionless enhancement by polarization transfer) experiments were performed using a transfer pulse of 135° to obtain positive signals for CH and CH_3 and negative ones for CH_2. Polarization transfer delays were adjusted to an average CH coupling of 135 Hz. Gradient versions of $^1H-^1H$ DQF-COSY (double quantum-filtered COSY), $^1H-^{13}C$ HSQC, and HMBC experiments were carried out using the standard pulse sequences as supplied by Bruker. Mass spectra were obtained from a VG Platform II Electrospray mass spectrometer performed using the negative-ion mode. HPLC analysis was performed on a Hewlett-Packard HP-1100 series using a reversed-phase column (LiChroCART 125-4, LiChrospher 100 RP-18) maintained at 30 °C with the following solvent program: solvent A, 2% AcOH in H_2O; solvent B, 2% AcOH in MeCN; starting from 4 up to 12% B in 20 min, to 20% B in 30 min and to 50% B in 45 min. The flow rate was 1 mL/min, and detection was made at 280 nm. Cellulose plates (Schleicher & Schnell) were used for TLC analysis ascending development with 6% aqueous acetic acid.

Plant Material. Cranberry fruit (*Vaccinium macrocarpon*) of the cultivar "Early Black" was collected at the Marucci Center for Blueberry and Cranberry Research, Chatsworth, NJ, in October 1996. "Early Black" is a common cultivated variety that is grown routinely for commercial purposes. Specimens of this variety are kept at the Marucci Center for Blueberry and Cranberry Research, which houses an extensive collection of cranberry germplasm.

Extraction and Isolation. Washed cranberry fruits (10 kg) were 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 the acetone, and the residue was diluted with water and defatted by extracting 3× with an equal volume of petroleum ether. The aqueous phase was reduced to a smaller volume and extracted 4× with an equal volume of ethyl acetate. The combined ethyl acetate extract was concentrated and the residue freeze-dried to yield a red syrupy material (47 g).

The syrupy residue (45 g) was dissolved in ethanol (40 mL) and chromatographed over a Sephadex LH-20 column (600 × 50 mm) using ethanol as the eluting solvent. Fractions (18 mL) were collected with a fraction collector and monitored by TLC developed with 6% aqueous acetic acid, and the plates were visualized by heating with a hair-dryer after spraying with an acidified solution of 5% vanillin in ethanol. On the basis of the TLC results, fractions were pooled to give predominantly anthocyanin, flavonoid glycosides, and three main (A−C) proanthocyanidin fractions. Chromatography of combined fraction A over a column of MCI Gel CHP 20P resin (500 × 25 mm) with methanol−water (3:7) yielded epicatechin (**1**) (R_f 0.33, 320 mg) and epicatechin-(4β→8)-epicatechin (**2**) (R_f 0.72, 410 mg). Fraction B was rechromatographed over Sephadex LH-20 with ethanol to yield epicatechin-(4β→8, 2β→*O*→7)-epicatechin (**3**) (R_f 0.28, 960 mg). Fraction C on chromatography over Sephadex LH-20 with ethanol−water (1:1) yielded epicatechin-(4β→6)-epicatechin-(4β→8, 2β→*O*→7)-epicatechin (**4**) (R_f 0.32, 110 mg), epicatechin-(4β→8, 2β→*O*→7)-epicat-

echin-(4β→8)-epicatechin (**5**) (R_f 0.30, 90 mg), and epicatechin-(4β→8)-epicatechin-(4β→8, 2β→*O*→7)-epicatechin (**6**) (R_f 0.45, 72 mg).

Epicatechin-(4β→6)-epicatechin-(4β→8, 2β→*O*→7)-epicatechin (4) was obtained as freeze-dried powder: $[\alpha]_D$ +105° (*c* 0.15, MeOH); UV (MeOH) λ_{max} (log ϵ) 282 nm (4.10); ^{1}H NMR (CD_3OD, δ) 2.6−3.3 (m, 2H-4^b), 3.97 (bs, H-3^t), 4.17 (bs, H-3^m), 4.28 (bs, H-3^b), 4.51(d, J = 3.2 Hz, H-4^m), 4.89 (obscured by H_2O, H-4^t), 4.94 (obscured by H_2O, H-2^b) 5.03 (bs, H-2^t), 6.08 (s, H-8^t), 6.09 (d, J = 2.0 Hz, H-8^t), 6.12 (H-6^t, H-6^b), 6.6−7.3 (H-2′, H-5′, H-6′ for t, m and b); ^{13}C NMR data, see Table 1; ESIMS *m*/*z* 863 [M − H]$^-$.

Acid-Catalyzed Degradation of 4 with Phloroglucinol. A mixture of the procyanidin trimer **4** (2 mg) and phloroglucinol (2 mg) was dissolved in 1% HCl in EtOH (1.0 mL), and the resulting solution was left at ambient temperatures for 24 h. The reaction mixture was evaporated to dryness under a stream of nitrogen, the residue was dissolved in MeOH, and the products were examined by HPLC. Epicatechin-(4β→2)-phloroglucinol and epicatechin-(4β→8, 2β→*O*→7)-epicatechin were detected as the products of the reaction.

P-Fimbriated *E. coli* Anti-adherence Activity. Detection of anti-adherence activity was accomplished using a material with a surface containing the putative disaccharide α-Gal(1→4)β-Gal receptor found on the exterior of uroepithelial cells.[22] This disaccharide sequence has a structure analogous to the P blood group antigen present on the surface of human red blood cells (HRBC).[23] Anti-adherence tests used in this study measured the capability of compounds **1**−**6** to suppress agglutination of either HRBCs (A_1, Rh+)[24] or latex beads coated with synthetic P receptor analogue[25] following incubation with P-fimbriated *E. coli.*

Escherichia coli strains were isolated from the urine of human patients diagnosed with urinary tract infections. Strains specific for P fimbrial adhesins were subcultured in tryptose broth at 37 °C for 16 h, transferred to colonization factor agar (CFA) plates,[24] 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 (PBS) solution at pH 7.0 at a concentration of 5×10^8 bacteria/mL. Compounds **1**−**6** were individually dissolved in 1 mL of PBS, neutralized with 1 N NaOH, and diluted serially (2-fold). 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, which were then incubated for 20 min on a rotary shaker at room temperature and evaluated microscopically for the ability to prevent agglutination. Controls included wells containing bacteria + PBS, HRBC or P-beads + PBS, bacteria + test compound, HRBC or P-beads + test compound, and bacetria + HRBC or P-beads.

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