DIMERIC PRODELPHINIDINS FROM *Limonium gmelinii* ROOTS. III.

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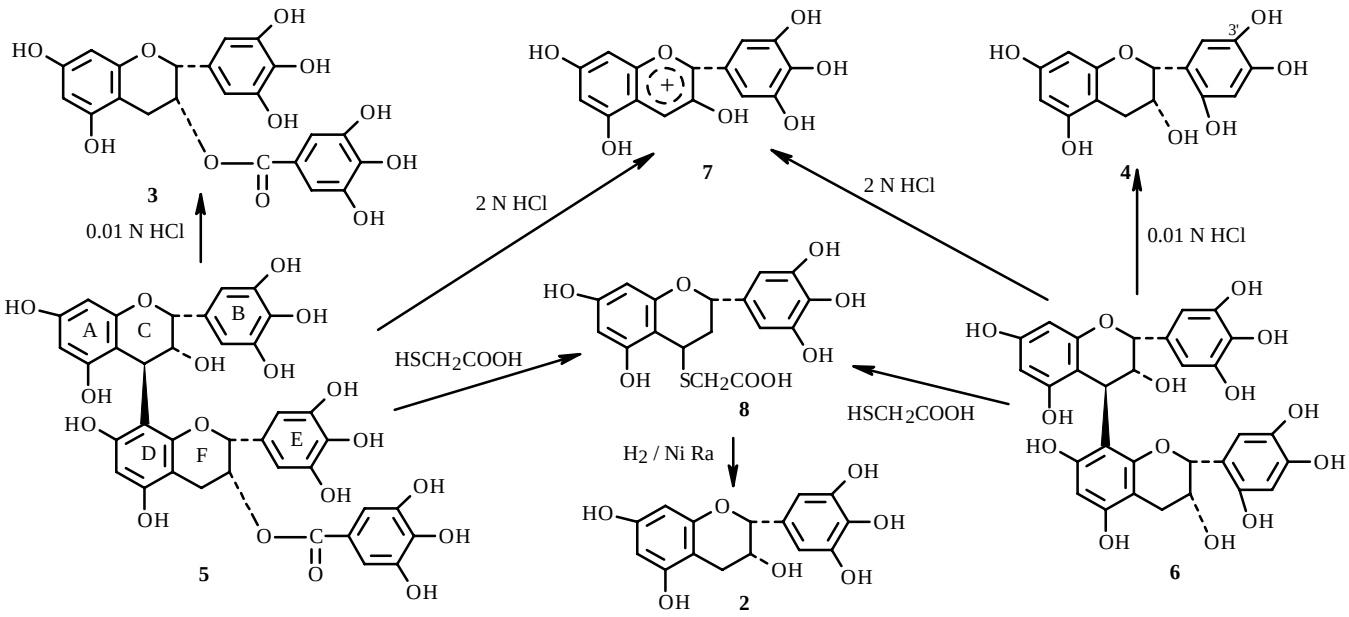
*Two dimeric proanthocyanidines identified as 2*R,*3*R,*4*R-*(-)-epigallocatechin-(4*β→*8)-2*R,*3*R-*(-)-epigallocatechin-3*-O-*gallate and 2*R,*3*R,*4*R-*(-)-epigallocatechin-(4*β→*8)-(-)-2*R,*3*R,*3,5,7,3′,4′,6′-hexahydroxyflavan were isolated by adsorption chromatography over polyamide of the ethylacetate fraction of the aqueous alcohol extract of* Limonium gmelinii *roots. The former proanthocyanidine was isolated for the first time from sea lavender whereas the latter is new.*

Key words: *Limonium gmelinii*, proanthocyanidines.

Dimeric proanthocyanidines in addition to monomeric flavans have been observed in *Limonium gmelinii* roots. The monomeric flavans were identified by physicochemical and spectral data as (+)-gallocatechin (**1**), (-)-epigallocatechin (**2**), (-)-epigallocatechin-3-*O*-gallate (**3**), and 3,5,7,3′,4′,6′-hexahydroxyflavan (**4**).



Proanthocyanidines were isolated by concentrating in vacuo the aqueous alcohol extract of *L. gmelinii* roots and then working up successively with ethylacetate and *n*-butanol. The ethylacetate fractions that contained a mixture of monomeric and dimeric flavans were chromatographed repeatedly over polyamide columns to isolate two pure dimeric proanthocyanidines **5** and **6**. Both compounds reacted with vanillin to give a red color typical of flavans, with iron (Fe^{3+}) salts, blue.

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TABLE 1. ^{13}C Chemical Shifts (ppm) of Flavans **5** and **6**

C atom	Compound **5**			Compound **6**	
	"upper" half	"lower" half	galloyl	"upper" half	"lower" half
2	75.1	77.3		75.0	78.5
3	72.6	69.5		71.8	66.2
4	37.0	25.1		37.2	29.3
6	97.0	96.8		96.7	96.9
8	96.5	107.4		95.2	107.2
10	103.5	103.5		101.0	101.7
5,7,9	153.3-154.6	153.3-154.6		155.3-157.5	155.3-157.5
1′	131.2	131.5	121.0	131.5	131.3
2′	106.6	106.6	109.9	108.0	117.0
3′	145.2	145.2	145.5	146.2	145.0
4′	133.2	133.2	137.7	134.5	146.5
5′	145.2	145.2	145.5	146.2	113.0
6′	106.6	106.6	109.9	108.0	146.0
$-COO^-$			165.3		

This was consistent with the presence of three adjacent phenol hydroxyls [1-3]. With KOH solution (50%), they were decomposed to phloroglucinol and gallic acid. Upon heating in HCl (2 N), anthocyanidine dye formed in both instances and was identified as delphinidin (**7**, λ_{max} 544 nm) [4-6]. In the presence of HCl (0.01 N) [4, 5], (-)-epigallocatechin-3-*O*-gallate (**3**) formed from the "lower" flavan unit of **5**; (-)-3,5,7,3′,4′,6′-hexahydroxyflavan (**4**), from the "lower" block of **6**. Thioethers (**8**) that were reduced by Raney nickel to (-)-epigallocatechin (**2**) were formed from the "upper" flavan units by decomposition with thioglycolic acid [7].

Molecular ions with $[M]^+$ 987 that corresponded with that calculated for the monoacetylpermethyl ether of **5** and with $[M]^+$ 834, with the diacetylpermethyl ether of **6**, confirmed that **5** and **6** were dimers. Thus, **5** and **6** were dimeric prodelphinidins.

The results were confirmed by ^{13}C NMR spectra of **5** and **6** (Table 1) and PMR spectra of their peracetyl derivatives.

The ^{13}C NMR spectrum of **5** showed C atoms C-5, C-7, and C-9 of a phloroglucinol ring, which had hydroxyls in both halves, at δ 153.3-154.6 ppm; C atoms C-6 and C-8, at 97.0 and 96.5 ppm, respectively [8-14]. Signals of C atoms of the side phenyl rings B and E were consistent with pyrogallol-type oxidation: C-2′ and C-6′ at δ 106.6 ppm, C-3′ and C-5′ at δ 145.2 ppm, and a characteristic signal of C-4′ at δ 133.2 ppm [8, 10, 12, 13]. Signals of the C-2 C atoms of both halves of the molecule were found at δ 75.1 and 77.3 ppm, respectively. This indicated that the asymmetric centers in both halves of the dimer had the 2,3-*cis*-configuration [8, 12, 14]. The substituted C atom C-4 of the "upper" half resonated at δ 37.0 ppm whereas the signal of unsubstituted C-4 of the "lower" flavan unit appeared at δ 25.1 ppm.

Substitution at C-4 causes C-2 to experience a strong negative γ-effect (Δδ -2.5 ppm); C-4, a significant positive α-effect (Δδ +8.2 ppm). This is characteristic of the β-orientation of the "lower" flavan unit on C-4 [8, 15, 16]. The spectrum contained signals for gallic acid that were assigned as follows: C-1″ at δ 121.0 ppm, C-2″ and C-6″, 109.9 ppm, C-3″ and C-5″, 145.5 ppm, C-4″, 137.7 ppm, and the carbonyl C atom at 165.3 ppm. Shifts to strong field for C-3 (δ 69.5 ppm) and C-4 (δ 25.1 ppm) were indicative of a gallyl ester on C-3 of the "lower" flavan unit [11, 17-19]. The resonances of C-8 and C-10 of the "lower" flavan unit at δ 107.4 and 101.5 ppm, respectively, indicated that there was a C-4-C-8 interflavan bond. If the bond were C-4-C-6, C-10 would have resonated at stronger field at δ 98.6-99.4 ppm [8, 10, 20]. These conclusions agreed with the mass and PMR spectra.

The PMR spectrum of the peracetyl derivative of **5** showed protons of heterocyclic C-2 of both halves as singlets at δ 5.58 and 4.78 ppm. This indicated that the protons on C-2 and C-3 were oriented *cis*-axial-equatorial [12, 14, 21-23]. Since the side phenyl ring in flavan-3-ols is equatorial [24], the C-2 proton is axial whereas the C3 proton is equatorial. A doublet for methylene protons with $J_{3,4}$ = 2 Hz (3.05 ppm, ring F) confirmed that C-3-H was equatorial. For and axial C-3-H, the C-4 methylene protons usually resonate as a multiplet owing to their magnetic nonequivalence. The C-4 proton of the "upper" flavan component gave a doublet with $J_{3,4}$ = 1 Hz at δ 4.46 ppm. Such a SSCC does not enable the configuration of C-4 to be

determined because it may be due to equatorial-equatorial or equatorial-axial arrangements of the H-3 and H-4 protons (ring C).

We propose calculating the contribution to the optical rotation of this center using the superposition principle [25] to determine the configuration at C-4. We found a trend for dimeric proanthocyanidins. The 4*R*-configuration gives a positive contribution; 4*S*, negative. The contribution to the optical rotation of C-4 was calculated from the difference of the optical rotation of **5** and the monomer components (-)-epigallocatechin and (-)-epigallocatechin-3-*O*-gallate: +70.2° -[(-57°) + (-182°)] = +309.2°. The positive contribution is consistent with the 4*R* configuration.

The PMR spectrum of the peracetyl derivative of the studied dimer contained signals for 13 aromatic (δ 2.27 and 2.02 ppm) and one aliphatic (δ 1.88 ppm) acetyl proton. Therefore, none of the five hydroxyls of the epigallocatechin nor the eight hydroxyls of the epigallocatechingallate are involved in the bonding. The bond also does not involve the side phenyl rings because two 2H singlets were recorded at δ 7.20 (ring B) and 7.00 ppm (ring E). This was confirmed by peaks in the mass spectrum of the monoacetylpermethyl ether of **5** with *m*/*z* 714 that formed through cleavage of an acetate and gallic acid from C-3 of both halves of the molecule and with *m*/*z* 404 and 181 that were characteristic of trimethoxy derivatives of the side phenyls [9, 26]. The appearance in the spectrum of signals for protons of a single methylene instead of two argued in favor of an interflavan bond at C-4.

The presence of three and not four protons on the phloroglucinol rings of **5** (δ 6.23 and 5.99 ppm for ring A; 6.40 ppm, ring D) indicated that one of the unsubstituted C atoms was involved in forming a bond. A 2H singlet that was assigned to H-2″ and H-6″ of the gallyl group was found at weak field at δ 7.62 ppm. Compared with the literature values [9, 21, 22, 27], this showed that the gallyl was located on C-3 of the "lower" flavan unit.

Thus, **5** was identified as 2*R*,3*R*,4*R*-(-)-epigallocatechin-(4β→8)-2*R*,3*R*-(-)-epigallocatechin-3-*O*-gallate on the basis of the chemical transformations, PMR, ^{13}C NMR, and mass spectra and a comparison with the literature [9, 14, 21].

The ^{13}C NMR and PMR spectra of **6** and its peracetyl derivative were similar to those of dimer **5**.

The PMR spectrum of **6** peracetate, like for **5**, showed C-2 protons of heterocycles in both halves of the molecule as singlets at δ 5.56 and 4.56 ppm. This indicated that the C-2 and C-3 protons were *cis*-axial-equatorial. The C-3 proton was also equatorial according to the doublet of methylene protons with $J_{3,4}$ = 1 Hz (δ 2.88 ppm). The C-4 proton of the "upper" flavan unit gave a doublet with $J_{3,4}$ = 1 Hz at δ 4.43 ppm. This did not enable the configuration at C-4 to be unambiguously determined. Like for **5**, the configuration of this C atom was determined using the superposition principle. The positive contribution to the optical rotation of C-4 in **6** that was calculated from the difference of its optical rotation and the components of the monomers (-)-epigallocatechin and (-)-3,5,7,3′,4′,6′-hexahydroxyflavan was consistent with the 4*R*-configuration: +60° - [(-57°) + (-35.2°)] = +152.2°.

The asymmetric centers of the heterocycles in both flavan units of **6** had the same 2*R*,3*R*-configuration. This was consistent with the C-2 resonances at δ 75.0 and 78.5 ppm. A negative γ-effect (Δδ -3.6 ppm) experienced by C-2 of the "upper" half of the molecule argued in favor of the β-orientation for the "lower" flavan unit. The interflavan bond was the same as C-4–C-8 in **5** according to the chemical shifts of C-8 (δ 107.2 ppm) and C-10 (101.7 ppm). The chemical shifts of the aromatic C atoms of the "upper" flavan unit of **6** were similar to those of the (-)-epigallocatechin block of **5**. The spectrum lacked signals for gallic acid. Resonances of the side phenyl ring E of the "lower" half of the molecule differed from those of ring B and were interpreted by us as follows (ppm): 131.3 (C-1′), 117.0 (C-2′), 145.0 (C-3′), 146.5 (C-4′), 113.0 (C-5′), 146.0 (C-6′).

Thus, the structure 2*R*,3*R*,4*R*-(-)-epigallocatechin-(4β→8)-(-)-2*R*,3*R*,3,5,7,3′,4′,6′-hexahydroxyflavan was proposed for **6** based on qualitative reactions, chemical transformations, and spectral analysis.

Such a structure for a dimeric prodelphinidin was established for the first time.

EXPERIMENTAL

Specific rotations were determined on a CM circular polarimeter; melting points, on a Kofler block; PMR spectra, on a Varian HA-100 spectrometer (100 MHz, $CDCl_3$, TMS internal standard, chemical shifts on the δ scale); ^{13}C NMR spectra, on a Bruker AMX-500 spectrometer [Me_2CO-d_6:D_2O (1:1)], mass spectra, in a M-1304 spectrometer.

Qualitative analysis was performed by paper chromatography (FN No. 3 and 4 paper) and TLC (Silufol plates, UV-254) using solvent systems butanol:acetic acid:water (BAW) (1, 40:12.5:29.2), acetic acid (2, 2%), acetic acid:HCl:H_2O (3, 30:3:10), and benzene:acetone (4, 8:2).

Isolation of Proanthocyanidins. Air-dried raw material (5 kg) was ground and worked up successively with benzene (3 L) and $CHCl_3$ (5 L) to remove lipophilic substances. Polyphenols were extracted by standing three times with aqueous ethanol (50%). The extract was condensed in vacuo on a water bath at 40-45°C. The resulting concentrate was diluted with water and extracted exhaustively with ethylacetate (6 L) until the reaction with vanillin was negative and then with butanol (4 L). The dry residue (41 g) of the ethylacetate extract was dissolved in acetone, mixed with polyamide, dried, placed on a polyamide column (560 × 30 mm), and eluted with $CHCl_3$, ethanol:$CHCl_3$, ethanol, and acetone with a gradient of water (30, 50, 70%). A total of 235 fractions of 150 mL volume was collected. Fractions 175-230 were chromatographed over a polyamide column (300 × 20 mm) to isolate the dimeric proanthocyanidins. Repeated chromatography isolated pure **5** (0.05 g) and **6** (0.065 g).

(-)-Epigallocatechin-(4β→8)-(-)-epigallocatechin-3-*O*-gallate (5), amorphous light-cream compound, $C_{37}H_{30}O_{18}$, $[\alpha]_D^{24}$ +70.2° (*c* 0.5, methanol), R_f 0.31 (1) and 0.32 (2). PMR of the peracetyl derivative of **5** (100 MHz, $CDCl_3$, δ, ppm, J/Hz): 1.88 (3H, s, aliph., OAc), 2.27 (36H, s, phenol., OAc), 2.02 (3H, s, phenol., OAc), 5.58 (s, H-2 C), 5.30 (m, H-3 C), 5.28 (m, H-3 F), 4.46 (d, $J_{3,4}$ = 1, H-4 C), 4.78 (br.s, H-2 F), 3.05 (d, $J_{4,3}$ = 2, H-4_{ax}, H-4_{eq}), 7.20 (s, H-2′ and H-6′ B), 7.00 (s, H-2′ and H-6′ E), 5.99 ($J_{6,8}$ = 2, H-6 A), 6.23 ($J_{8,6}$ = 2, H-8 A), 6.40 (s, H-6 D), 7.62 (s, H-2″, H-6″, gallyl). Table 1 lists the ^{13}C NMR spectrum of **5**.

Alkaline Cleavage. Compound **5** (2 mg) was boiled with KOH solution (1 mL, 50%) for 20 min, cooled, and acidified with H_2SO_4 solution (25%). The cleavage product was extracted with ethylacetate and identified by comparison with authentic samples as phloroglucinol [R_f 0.71 (1), 0.42 (2)] and gallic acid [R_f 0.64 (1), 0.50 (2)].

Preparation of Anthocyanidin. Compound **5** (2 mg) was dissolved in HCl (1 mL, 2 N), refluxed on a boiling-water bath for 20 min, and diluted with water. The product was extracted with isoamyl alcohol. Chromatography of the dye that was formed with authentic samples of anthocyanidins using system 3 showed that it was identical to delphinidin [R_f 0.36 (3)].

Acid Cleavage. Compound **5** (2 mg) was refluxed with HCl (0.01 N, 30 min) on a boiling-water bath. The flavan that was formed was identified by comparison with authentic samples as (-)-epigallocatechin-3-*O*-gallate [R_f 0.63 (1), 0.27 (2)].

Thiolytic Cleavage. Compound **5** was dissolved in ethanol (1 mL) and heated with thioglycolic acid (1 mL, 25%) on a boiling water bath for 1 h. The cleavage was monitored by paper chromatography. The reaction products contained a flavan-3-ol, which was identified as (-)-epigallocatechin-3-*O*-gallate (**3**). The reaction mixture was treated with a saturated solution of $NaHCO_3$ until it was basic. The flavan that was formed was extracted exhaustively with ethylacetate. Then the reaction mixture was acidified with HCl. The thioether was extracted with ethylacetate. Reduction of the latter over Raney nickel formed (-)-epigallocatechin [R_f 0.40 (1), 0.39 (2)].

Preparation of Peracetyl Derivative. Compound **5** (30 mg) that was dried over P_2O_5 was dissolved in absolute pyridine (3 mL) and acylated by freshly distilled acetic anhydride (6 mL) for 130 h at room temperature. The reaction mixture was poured into icewater and left at +5°C for 12 h. The resulting precipitate was filtered off, washed with water, and dried in a vacuum desiccator over P_2O_5. The acetyl derivative was purified over silica-gel:chromaton (5:1) columns with elution by benzene, benzene:$CHCl_3$ (2:1, 1:1, 1:2), and $CHCl_3$ to afford the peracetate (20 mg), the purity of which was monitored by TLC using system 4.

Preparation of Monoacetylpermethyl Ether. The peracetate (20 mg) was dissolved in methanol (5 mL) and treated dropwise with constant stirring so that it did not overheat with dimethylsulfate (4 mL) and KOH solution (6 mL, 50%). After the reaction was complete, the mixture was poured into icewater and left at +5°C for 3 h. The products were extracted with $CHCl_3$. After the solvent was removed, the dry residue was dissolved in absolute pyridine (2 mL) and acylated with acetic anhydride (5 mL) for 72 h at room temperature. It was purified analogously to the peracetate.

(-)-Epigallocatechin-(4β→8)-(-)-3,5,7-3′,4′,6′-hexahydroxyflavan (6), amorphous light-brown compound, $C_{30}H_{26}O_{14}$, $[\alpha]_D^{22}$ +60° (*c* 0.5, methanol), R_f 0.26 (1), 0.37 (2).

Peracetyl derivative of 6, amorphous white compound, $C_{54}H_{50}O_{26}$, $[\alpha]_D^{20}$ +45° (*c* 0.4, ethanol), R_f 0.45 (TLC, 4). PMR of the peracetate of **6** (100 MHz, $CDCl_3$, δ, ppm, J/Hz): 2.19 (24H, s, phenol., OAc), 2.03 (3H, s, phenol., OAc), 1.90 and 1.81 (6H, s, aliph., OAc), 5.56 (s, H-2 C), 4.43 (d, $J_{3,4}$ = 1, H-4 C), 4.56 (s, H-2 F), 5.15 (m, H-3 C, F), 2.88 (d, J = 1, H-4_{ax}, H-4_{eq}, F), 6.3 ($J_{8,6}$ = 2, H-8 A), 6.01 ($J_{6,8}$ = 2, H-6 A), 6.50 (s, H-6 D), 7.21 (s, H-2′, H-6′ B), 6.91-7.12 (H-2′, H-5′ E). Table 1 lists the ^{13}C NMR spectrum of **6**. Mass spectrum (*m*/*z*, %): 834 (50) $[M]^+$, 774 (95), 522 (50), 357 (58), 355 (43), 252 (40), 210 (32), 181 (23).

Compound **6** was studied using the same methods as for **5**.

Alkaline Cleavage. Compound **6** (2 mg) was heated with KOH solution (50%). The cleavage products were identified as phloroglucinol and gallic acid.

Preparation of Anthocyanidine. Compound **6** (2mg) was heated with HCl (2 N). The anthocyanidin that was formed was identified as delphinidin.

Acid Cleavage. Compound **6** (2 mg) was heated with HCl (1 mL, 0.01 N). The flavan was formed and identified as (-)-epigallocatechin.

Thiolytic Cleavage. A flavan identical to (-)-3,5,7,3′,4′,6′-hexahydroxyflavan was formed from the "lower" flavan unit. A thioether was formed from the "upper" unit and was reduced with Raney nickel to (-)-epigallocatechin.

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