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PROCYANIDIN DIMERS AND TRIMERS FROM DOUGLAS FIR INNER BARK

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Key Word Index—*Pseudotsuga menziesii*; Pinaceae, flavan-3-ols, procyanidins; dimers, trimers, benzylthiol, degradation.

Abstract—The aqueous fraction of the inner bark extract of Douglas fir (*Pseudotsuga menziesii*) yielded two novel natural procyanidin trimers, epicatechin-(4β→8)-catechin-(4α→8)-catechin and epicatechin-(4β→6)-epicatechin-(4β→8)-epicatechin, and two known trimers, epicatechin-(4β→8)-epicatechin-(4β→8)-epicatechin and epicatechin-(4β→8)-epicatechin-(4β→8)-catechin. Catechin, epicatechin and procyanidin dimers B1, B2, B3 and B4 were also isolated. The structures of all of these procyanidins were elucidated from their ^{13}C NMR data and by partial acid-catalysed degradation with benzylthiol.

INTRODUCTION

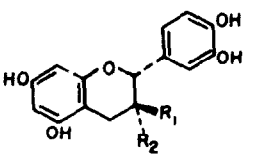
The forest products industry in Oregon, U.S.A., generates about three million tons of waste bark from Douglas fir (*Pseudotsuga menziesii* Mirb. Franco) trees alone [1]. Although several studies [2–5] of the chemistry of this renewable resource have been made with the object of facilitating its utilization, none has addressed in any detail the nature of the condensed tannins or procyanidins that are generally present in great abundance in bark. In an earlier communication [6], the isolation and identification of eight flavanoid glycosides from the inner bark of Douglas fir was reported. The present paper deals with the nature of the procyanidin dimers and trimers that occur with these glycosides.

RESULTS AND DISCUSSION

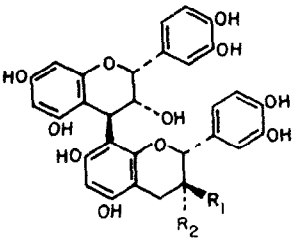
Column chromatography of the procyanidin portion [6] of the water-soluble fraction of the inner bark on Sephadex LH-20 and MCI-gel CHP-20P yielded (+)-catechin (**1**), (−)-epicatechin (2), and four procyanidin dimers: B1, epicatechin-(4β→8)-catechin (**3**); B2, epicatechin-(4β→8)-epicatechin (**4**); B3, catechin-(4α→8)-catechin (**5**); and B4, catechin (4α→8)-epicatechin (**6**). All were identified by FABMS and ^{13}C NMR, and their identities were confirmed by comparison with authentic samples on cellulose 2-D TLC. In addition, four procyanidin trimers, (**7**) to (**10**), were isolated in relatively good yields.

Compound **7** yielded cyanidin on heating with 5% HCl in *t*-BuOH, and FABMS gave an $[M-H]^-$ ion peak at *m/z* 865, indicating the compound to be a trimeric procyanidin. This constitution was supported by ^{13}C NMR data showing aromatic carbon resonances diagnostic of the phloroglucinol A-ring and the catechol B-ring [7]. The upfield position of the C-2 resonances (δ76.9 and 77.1) clearly established the 2,3-*cis* configuration for both flavanoid extender units, and the remaining C-2 resonance at δ81.8 suggested that catechin was the terminal unit [7]. Further evidence for this structural assignment and for the mode of interflavanoid linkages was obtained by partial acid-catalysed cleavage with benzylthiol (Scheme 1). Catechin (**1**), epicatechin (4β→S)-benzylthioether (**11**), the procyanidin dimer epicatechin-(4β→8)-catechin (**3**), and epicatechin-(4β→8)-epicatechin-(4β→S)-benzylthioether (**13**) were generated by reaction of **7** with benzylthiol in ethanol in the presence of acetic acid. The upper interflavanoid linkage in **7** was retained in the dimer thioether (**13**) and the lower bond was preserved in the dimer (**3**), showing that the flavanoid units were originally linked by a C-4 to C-8 interflavanoid bond. The generation of catechin (**1**) and the dimer (**3**) confirmed the identity of the terminal unit and established **7** as epicatechin-(4β→8)-epicatechin-(4β→8)-catechin. The structure was further confirmed by comparing its spectroscopic and chromatographic data with the authentic trimer, isolated previously from reaction of catechin with *Pinus taeda* polymer [8].

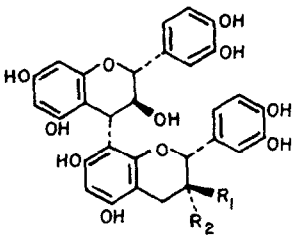
Compound **8**, obtained in highest yield of the lower oligomeric procyanidins, reacted with 5% HCl in *t*-BuOH to generate cyanidin, and FABMS gave an $[M-H]^-$ ion peak at *m/z* 865, indicating **8** also was a procyanidin trimer. The presence of a single band at 1520 cm^{-1}, the region attributed to the skeletal stretching modes of the aromatic ring in the IR spectrum of **8**, corroborated the all-catechol B-ring functionality of the flavan units [9]. In addition, the absorption band in the 795–800 cm^{-1} region was apparently stronger for **8** than for **7**, indicating 2,3-*cis* stereochemistry for all flavan units [9]. Evidence supporting this stereochemical assignment was also available from the ^{13}C NMR spectrum of the compound (Table 1), in which the C-2 resonances were all located in the upfield region (δ77.1 to 79.7). Partial degradation of **8** with benzylthiol gave epicatechin (**2**), epicatechin-(4β→S)-benzylthioether (**11**), epicatechin-(4β→8)-epicatechin (**4**) and epicatechin-(4β→8)-epicatechin (4β→S)-benzylthioether (**13**). Compound **8** therefore was the all-(C-4 to C-8)-linked procyanidin trimer epicatechin-(4β→8)-epicatechin-(4β→8)-epi-



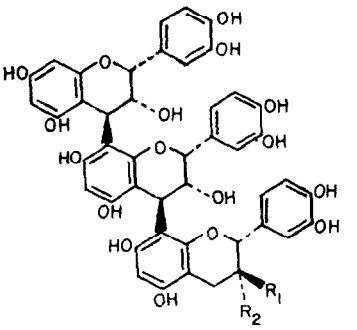
(1) R_1 = OH, R_2 = H
(2) R_1 = H, R_2 = OH



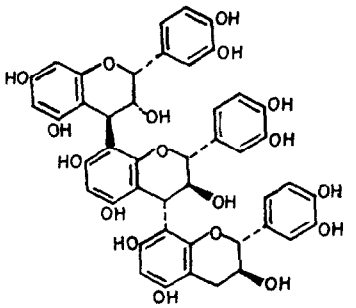
(3) R_1 = OH, R_2 = H
(4) R_1 = H, R_2 = OH



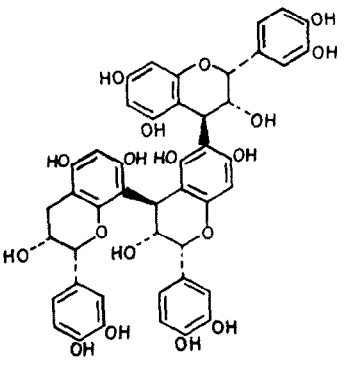
(5) R_1 = OH, R_2 = H
(6) R_1 = H, R_2 = OH



(7) R_1 = OH, R_2 = H
(8) R_1 = H, R_2 = OH



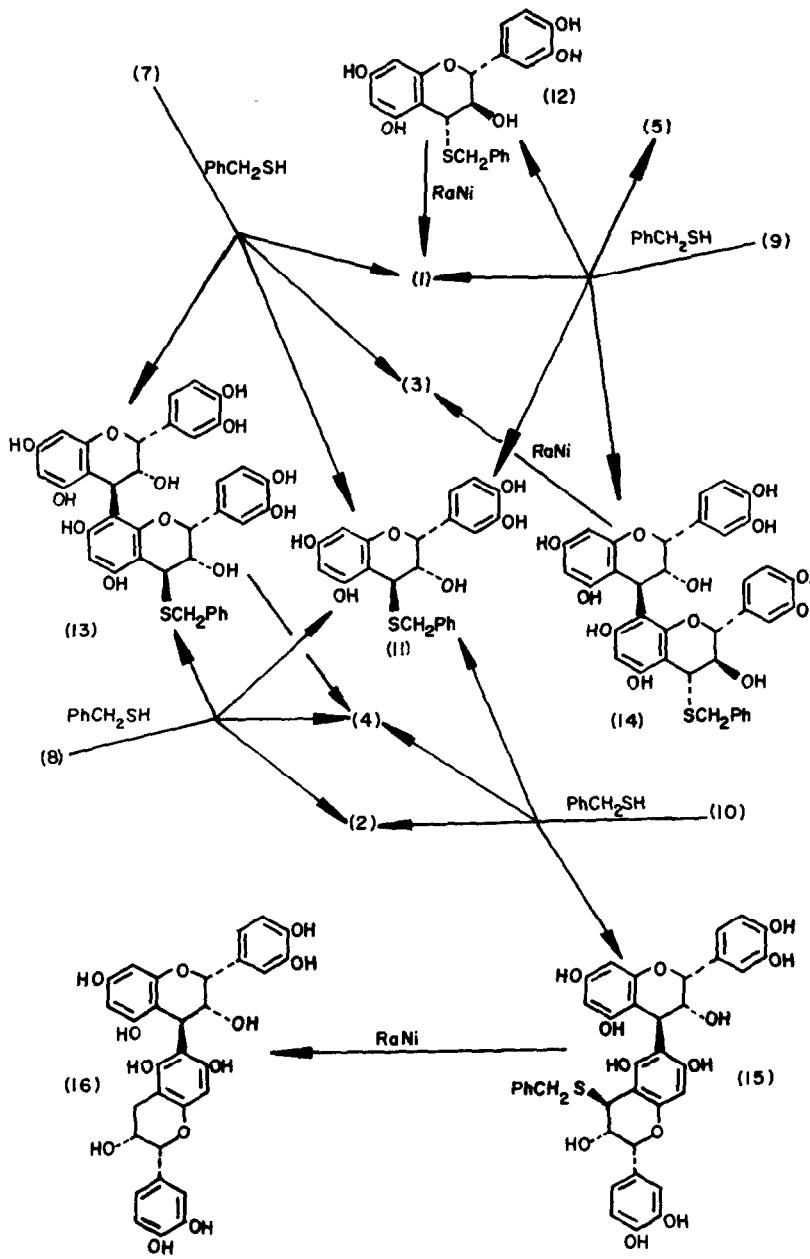
(9)



(10)

catechin. This constitution was confirmed by chromatographic (2-D TLC) and spectroscopic comparison with authentic material obtained previously by reaction of epicatechin with *Aesculus hippocastanum* tannins [8].

Compound **9** also was shown to be a procyanidin trimer by cyanidin generation with 5% HCl and by an $[M-H]^-$ ion peak at *m/z* 865 by FABMS The 795–800 cm^{-1} band in the IR spectrum was observed as a shoulder and was much weaker than that of **7** or **8**, indicating the 2,3-*trans* configuration for the majority of the flavonoid units [9]. The ^{13}C NMR spectrum of **9** showed atropisomerism with consequential multiplicity of carbon resonances Similar multiplicity of signals was also observed in the ^{13}C NMR spectra of the procyanidin dimers catechin-(4α→8)-epicatechin (**3**) (rotomer ratio 1:3 2) and catechin-(4α→8)-catechin (**4**) (rotomer ratio 1·1.2 in MeOH-d_4). In such compounds, where the 2,3-*trans* procyanidin unit carries the appending lower flavanoid unit in the pseudo-equatorial orientation, a steric barrier to rotation about the interflavanoid bond exists [10–12]. The possibility that the multiplicity of carbon resonances in the spectrum of **9** resulted from an impurity was ruled out by the following observations. Repetitive purification by column chromatography on MCI-gel and Sephadex LH-20 did not produce any changes in the shape or relative size of the carbon signals, and the first and last cuts of the chromatographic peak showed the same ^{13}C NMR spectrum as the entire peak Finally, 2-D TLC consistently showed only one spot. The C-2 resonances at δ75 9 and 84.3 in the ^{13}C NMR spectrum of **9** indicated the two extending flavanoid units possessed the contrasting 2,3-*cis* and 2,3-*trans* stereochemistries The terminating C-2 resonance at *ca* δ81 suggested the



Scheme 1. Partial degradation of trimers **7**–**10** with benzylthiol ($PhCH_2SH$).

end group to be catechin; hence the ^{13}C MNR data were consistent with IR data implying the 2,3-*trans* configuration for the majority of the flavanoid units. The sequence of the flavanoid units in the trimeric chain and the nature of the interflavanoid linkage were established by partial degradation with benzylthiol (Scheme 1). Treatment of **9** with benzylthiol in presence of HOAc in EtOH gave catechin (**1**) as the only monomer, confirming it to be the bottom terminal unit. Catechin-(4α→8)-catechin (**5**) was generated as the only procyanidin dimer, which defined the lower two units as having the 2,3-*trans* stereochemistry and being linked by a C-4 to C-8 interflavanoid bond. Epicatechin-(4β→S)-benzylthioether (**11**) and catechin-(4α→S)-benzylthioether (**12**) were also produced; the identity of the latter compound was confirmed by chromatographic comparison with the thioether generated by treating catechin-(4α→8)-catechin (**5**) with benzylthiol. These results established that the 2,3-*cis* extender unit in **9** had to be at the top. This sequence and the remaining linkage was established by identification of the dimeric procyanidin benzylthioether (**14**). Isolating **14** by multiple 2-D TLC and exposing the eluted compound to Raney nickel in EtOH gave epicatechin-(4β→8)-catechin (**3**), establishing **9** as epicatechin-(4β→8)-catechin-(4α→8)-catechin, a new natural product.

Compound **10** similarly gave cyanidin and an $[M-H]^-$ ion peak at *m/z* 865, showing it too was a procyanidin trimer. The IR spectrum of **10**, like that of **8**, showed relatively strong absorption in the 795–800 cm^{-1} region, indicating 2,3-*cis* stereochemistry for all flavanoid

Table 1 ^{13}C NMR chemical shifts of procyanidin trimers in MeOH-d_4

Compound	Unit*	C-2	C-3	C-4	C-6	C-8
Epicatechin-(4β→8)-epicatechin-(4β→8)-catechin (**7**)	T	77 1	73 3	37 2	97 4	96 2
	M	76 9	72 2	37 2	96 6	106.7
	B	81 8	68 1	26 7	97 4	108 0
Epicatechin-(4β→8)-epicatechin-(4β→8)-epicatechin (**8**)	T	77 1	73 4	37 4	96 7	96 3
	M	77.1	72.9	37 4	97 6	107 2
	B	79 7	66 8	29 7	97 7	107 6
Epicatechin-(4β→8)-catechin-(4α→8)-catechin (**9**)	T	75 8	72.6	37 0	95 9	95 9
	M	84 2	73 7	38 7	97 7	108 0
	B	81 0	68 4	28 4	95.9	108 6
Epicatechin-(4β→6)-epicatechin-(4β→8)-epicatechin (**10**)	T	77 4	73 3	37 3	96 6	96 1
	M	77 1	73 1	37.4	108 6	96 6
	B	79 5	66 9	29 7	97 3	106.6

* T = top, M = middle, B = bottom unit

units; these data were consistent with ^{13}C NMR data, in which the C-2 resonances were all located upfield (< 80 ppm). Partial degradation of **10** with benzylthiol produced epicatechin (**2**), epicatechin-(4β→S)-benzylthioether (**11**), epicatechin-(4β→8)-epicatechin (**4**) and epicatechin-(4β→6)-epicatechin-(4β→S)-benzylthioether (**15**) The last-mentioned compound was identified by TLC comparison with authentic material [8] and by reaction with Raney nickel to yield procyanidin B5 (**16**) Hence **10** was another new natural procyanidin trimer from Douglas fir: epicatechin-(4β→6)-epicatechin-(4β→8)-epicatechin. This trimer has been obtained synthetically by hydrolysing rhubarb procyanidin with tannase [13].

Isolation of these lower M_r polyphenols in relatively high yields from the aqueous fraction that had already been extracted repeatedly with ethyl acetate was unexpected. Also unexpected was the overwhelming presence in this procyanidin fraction of C-4 to C-8 interflavanoid linkages, to the almost total exclusion of C-4 to C-6 bonds. The aqueous extract also contained *ca* 47% carbohydrate by weight [6], with the balance predominantly higher procyanidin oligomers. These materials conceivably could participate in complex formation with the lower molecular weight polyphenols to inhibit their extraction with ethyl acetate. Dreiding models suggest that the molecular shapes of procyanidins with C-4 to C-6 linkages differ from their more common regioisomers. Possibly they complex less effectively with carbohydrate and with other linear procyanidin polymers and so are more readily extracted by EtOAc Evidence of this has been suggested by our preliminary studies of the EtOAc extract, which indicate presence of C-4 to C-6 linked procyanidin dimers

It has been suggested that chain propagation in procyanidin synthesis occurs by condensation of free flavan-3-ols with flavan carbocations [14] or quinone-methides [15, 16] to dimers, which give rise in turn to higher oligomers. The presence of (+)-catechin (**1**) and (−)-epicatechin (**2**) in Douglas fir bark would suggest the co-occurring procyanidins would terminate with these entities. Furthermore, the isolation of B1 (**3**), B2 (**4**), B3 (**5**), and B4 (**6**) indicates that they should be accompanied by higher oligomers with these units as the lower end of the procyanidin chain. With the exception of B4, these units were indeed represented in the co-occurring procyanidin trimers **7**–**10**

EXPERIMENTAL

^{13}C NMR spectra were obtained in MeOH-d_4 FABMS were obtained on samples dissolved in a matrix of a 5 1 mixture of dithiothreitol and dithioerythritol (Magic Bullet) Analytical and semi-prep TLC were performed on Schleicher and Schuell cellulose plates containing luminescer (254 nm) and developed with *t*-BuOH–HOAc–H_2O (3 1 1, solvent A) and HOAc–H_2O (3 47, solvent B)

Extraction and isolation Fresh inner bark (1 kg) of a 120-year-old Douglas fir was extracted exhaustively with MeOH, and the extract was concd on a rotary evaporator under red pres. The residual extract was diluted with H_2O and washed exhaustively, first with hexane and then with EtOAc, before freeze-drying. A fluffy brown solid (48 g) was obtained, 40 g of which was applied to a Sephadex LH-20 column (5 × 25 cm) Washing the column with MeOH–H_2O (1 1) yielded 3 main fractions: carbohydrate (19 0 g), flavanoid (2 2 g), and oligomeric procyanidin (9 0 g) CC of the procyanidin fraction, alternating between Sephadex LH-20 (EtOH–H_2O 19 1 to 3 17) and MCI-gel CHP-20P (MeOH–H_2O 3 7), was repeated until chromatographically homogeneous products were obtained

Degradation with benzylthiol Benzylthiol (4 mg), and HOAc (2 drops) were added to a sample of procyanidin (2 mg) in EtOH (2 ml) in a vial The vial was flushed with N_2 for 1 min, sealed and heated at 95° for 1 hr Reaction products were analysed by 2-D TLC developed with solvents A and B and were visualised by spraying with vanillin–HCl For semi-preparative TLC, 10 or more plates were used, spots were visualized under UV, scraped, transferred to pasteur pipettes, and eluted with MeOH The eluant was concd under a stream of N_2 and treated with Raney nickel for 15 min in the presence of a drop of HOAc The products were re-examined by 2-D TLC

(+)-catechin (**1**) Freeze-dried powder (7 mg), $[\alpha]_{589}$ +20° (MeOH, *c* 0 1), R_f 0 68 (A), 0 50 (B) ^{13}C NMR (ppm) 28 5 (C-4), 68 8 (C-3), 82 8 (C-2), 95 5 (C-8), 96 3 (C-6), 100 8 (C-4a), 115 3 (C-2'), 116 1 (C-5'), 120 0 (C-6'), 132 2 (C-1'), 146 2 (C-3', C-4'), 156 9, 157 6 and 157 8 (C-5, C-7, C-8a)

(−)-*Epicatechin* (**2**). Freeze-dried powder (25 mg), $[\alpha]_{589}$ −43° (MeOH; *c* 0.15), R_f 0.50 (A), 0.30 (B). ^{13}C NMR (ppm): 29.2 (C-4), 67.5 (C-3), 79.9 (C-2), 95.9 (C-8), 96.4 (C-6), 100.1 (C-4a), 115.3 (C-2′), 115.9 (C-5′), 119.4 (C-6′), 132.3 (C-1′), 145.8, 145.9 (C-3′, C-4′), 157.4, 157.7 and 158.0 (C-5, C-7, C-8a).

Epicatechin-(4β→8)-*catechin* or B1 (**3**) Freeze-dried powder (562 mg), $[\alpha]_{589}$ −320° (MeOH; *c* 0.1), R_f 0.42 (A) and 0.60 (B). FABMS gave an $[M-H]^-$ peak at *m/z* 577. ^{13}C NMR (ppm): 28.6, 68.4, 73.0, 76.9, 82.2, 95.8, 96.1, 97.0, 101.1 (2×), 107.8, 115.3, 115.9 (2×), 116.1, 119.3, 119.6, 132.2, 132.7, 145.4 (2×), 145.7 (2×), 155.5–157.5 Treatment with benzylthiol yielded **1** and **11**

Epicatechin-(4β→8)-*epicatechin* or B2 (**4**). Freeze-dried powder (302 mg), $[\alpha]_{589}$ +29.2° (MeOH, *c* 0.15), R_f 0.43 (A) and 0.63 (B) FABMS gave an $[M-H]^-$ peak at *m/z* 577 ^{13}C NMR (ppm): 29.6, 37.1, 67.0, 73.5, 77.1, 79.7, 96.1, 96.5, 97.3, 100.5, 101.4, 115.3 (2×), 115.9 (2×), 119.3 (2×), 132.1, 132.6, 145.6 (2×), 145.8 (2×), 154.5, 156.4 (2×), 157.8, 158.3 (2×) Reaction with benzylthiol gave **2** and **11**

Catechin-(4α→8)-*catechin* or B3 (**5**). Freeze-dried powder (74 mg), $[\alpha]_{589}$ −93° (MeOH; *c* 0.09), R_f 0.37 (A) and 0.50 (B). FABMS gave an $[M-H]^-$ peak at *m/z* 577 ^{13}C NMR (ppm, *rotomer peaks) 28.5*, 28.7, 38.6, 68.7*, 68.9, 73.7, 82.4, 82.9*, 83.9, 84.1*, 96.0, 96.2*, 96.8, 97.3, 97.5*, 101.0, 102.2, 107.2, 108.1, 108.3*, 115.1–116.2, 119.9, 120.1*, 120.6, 120.9*, 131.8, 132.0*, 132.4*, 132.6, 145.4–145.7, 154.8–158.6. Reaction with benzylthiol gave **1** and **12**.

Catechin-(4α→8)-*epicatechin* or B4 (**6**). Freeze-dried powder (69 mg), $[\alpha]_{589}$ −6.0° (MeOH; *c* 0.14), R_f 0.53 (A) and 0.54 (B). FABMS gave an $[M-H]^-$ peak at *m/z* 577. ^{13}C NMR (ppm, *rotomer peaks) 29.4*, 30.1, 38.8*, 38.9, 67.4, 67.8*, 73.8, 73.8*, 79.9*, 80.0, 83.9, 84.0*, 96.2, 96.5*, 97.2*, 97.6 (2×), 97.7, 99.5, 101.5*, 107.2, 107.4*, 108.3*, 108.7, 114.8–116.5, 119.2, 120.3*, 120.5*, 121.2, 131.7–132.6, 145.6–146.5, 155.4–158.7. Reaction with benzylthiol gave **2** and **12**.

Epicatechin-(4β→8)-*epicatechin*-(4β→8)-*catechin* (**7**). Light brown freeze-dried powder (220 mg), $[\alpha]_{589}$ +40° (MeOH; *c* 0.12), R_f 0.27 (A) and 0.55 (B). FABMS gave an $[M-H]^-$ peak at *m/z* 865. ^{13}C NMR (ppm): 26.7, 37.2, (2×), 68.1, 72.2, 73.3, 76.9, 77.1, 81.8, 96.2 (2×), 96.6, 97.4, 100.9, 101.3, 102.4, 106.7, 108.0, 114.5, 115.1, 116.0, (2×), 116.2 (2×), 118.9, 119.4 (2×), 132.5 (3×), 145.2–145.9, 153.7–158.2. IR ν_{max}^{KBr} (cm^{-1}): 3416 (b), 1612, 1520, 1446, 1365, 1285, 1248, 1210, 1150, 1107, 1061, 820, 797, 779. Reaction with benzylthiol gave **1**, **11**, **13** and **3**.

Epicatechin-(4β→8)-*epicatechin*-(4β→8)-*epicatechin* (**8**). Light brown freeze-dried powder (470 mg), $[\alpha]_{589}$ +80° (MeOH; *c* 0.16), R_f 0.25 (A) and 0.54 (B). FABMS gave $[M-H]^-$ peak at *m/z* 865. ^{13}C NMR (ppm). 29.7, 37.4, 66.8, 72.8, 73.4, 77.0 (2×), 79.7, 96.2, 96.6, 97.6, 97.7, 100.6, 101.2, 102.0, 107.3, 107.8, 115.1–116.0, 119.0,–119.2, 132.1, 132.5, 132.6, 145.5–145.9, 156.5, 158.2. Reaction with benzylthiol gave **2**, **11**, **13** and **4**.

Epicatechin-(4β→8)-*catechin*-(4α→8)-*catechin* (**9**). Light brown freeze-dried powder (207 mg), $[\alpha]_{589}$ −180° (MeOH; *c* 0.10), R_f 0.17 (A) and 0.64 (B). FABMS gave an $[M-H]^-$ peak at *m/z* 865 ^{13}C NMR (ppm, *rotomer peaks) 28.6, 37.1, 38.8, 67.0* (b), 68.5, 72.6, 73.8, 75.9, 76.7*, 81.0 (b), 83.0*, 84.3, 95.9 (b), 97.4 (b), 101.0 (b), 103.8 (b), 108.1, 108.7, 109.8* (b), 115.2–116.6, 119.3–121.2, 132.1, 132.6, 133.0, 145.1–146.2, 154.9–157.3. Reaction with benzylthiol gave **1**, **12**, **11**, **14** and **5**. The constitution of **14** was confirmed by isolating the compound by semi-prep. 2-D TLC and reacting the isolate with Raney Ni to give **3**.

Epicatechin-(4β→6)-*epicatechin*-(4β→8)-*epicatechin* (**10**). Light brown freeze-dried powder (46 mg), $[\alpha]_{589}$ +79° (MeOH; *c* 0.08), R_f 0.30 (A) and 0.57 (B). FABMS gave an $[M-H]^-$ peak at *m/z* 865. ^{13}C NMR (ppm): 29.7, 37.3, 37.5, 66.9, 73.1, 73.3, 77.1, 77.4, 79.5, 96.1, 96.6 (2×), 97.3, 100.1, 100.6, 100.4, 101.4, 106.5, 108.6, 115.1–115.9, 118.8, 119.2, 119.6, 132.1, 132.3, 132.6, 145.3, 145.5, 145.6, 145.8 (2×), 146.0, 155.4–159.2. Reaction with benzylthiol gave **2**, **11**, **15** and **4**. The identity of **15** was confirmed by isolation by semi-prep. TLC and reaction of the isolate with Raney Ni to yield (**16**).

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