Plant Proanthocyanidins. Part 5.[1] Sorghum Polyphenols

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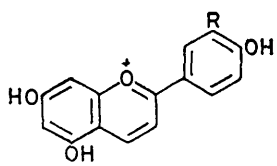
The polymeric procyanidin from the seed coat of sorghum grain has been isolated. Chemical studies show that it has a molecular weight in the region 1 700—2 000 and that it is derived biosynthetically from the condensation of the flavanol carbocation (5) (chain extender) and (+)-catechin (3) (chain initiator).

SORGHUM is an important food grain in many tropical countries. Its nutritional quality is considerably diminished in many hybrids by the presence of ' tannins ' and when the grain is disrupted by milling the polyphenols inhibit endogeneous enzymes in aqueous suspensions and reduce the brewing quality of the ground malt.[2] The presence of ' tannins ' in a cereal is unusual (barley is the only other in which they are found) and as a preliminary to the evaluation of their effect on the nutritional value of the grain the chemical nature of these polyphenols has been determined. In earlier work glycosylated forms of the 3-deoxyanthocyanidins luteolinidin (1) and apigenidin (2) have been shown to be present in sorghum [3] and Bate-Smith has presented evidence relating to the unique occurrence of a flavan-4-ol (luteoforol) in the grain.[4] Strumeyer and Malin have obtained the tannins from Leoti sorghum and shown that these have the properties of oligomeric



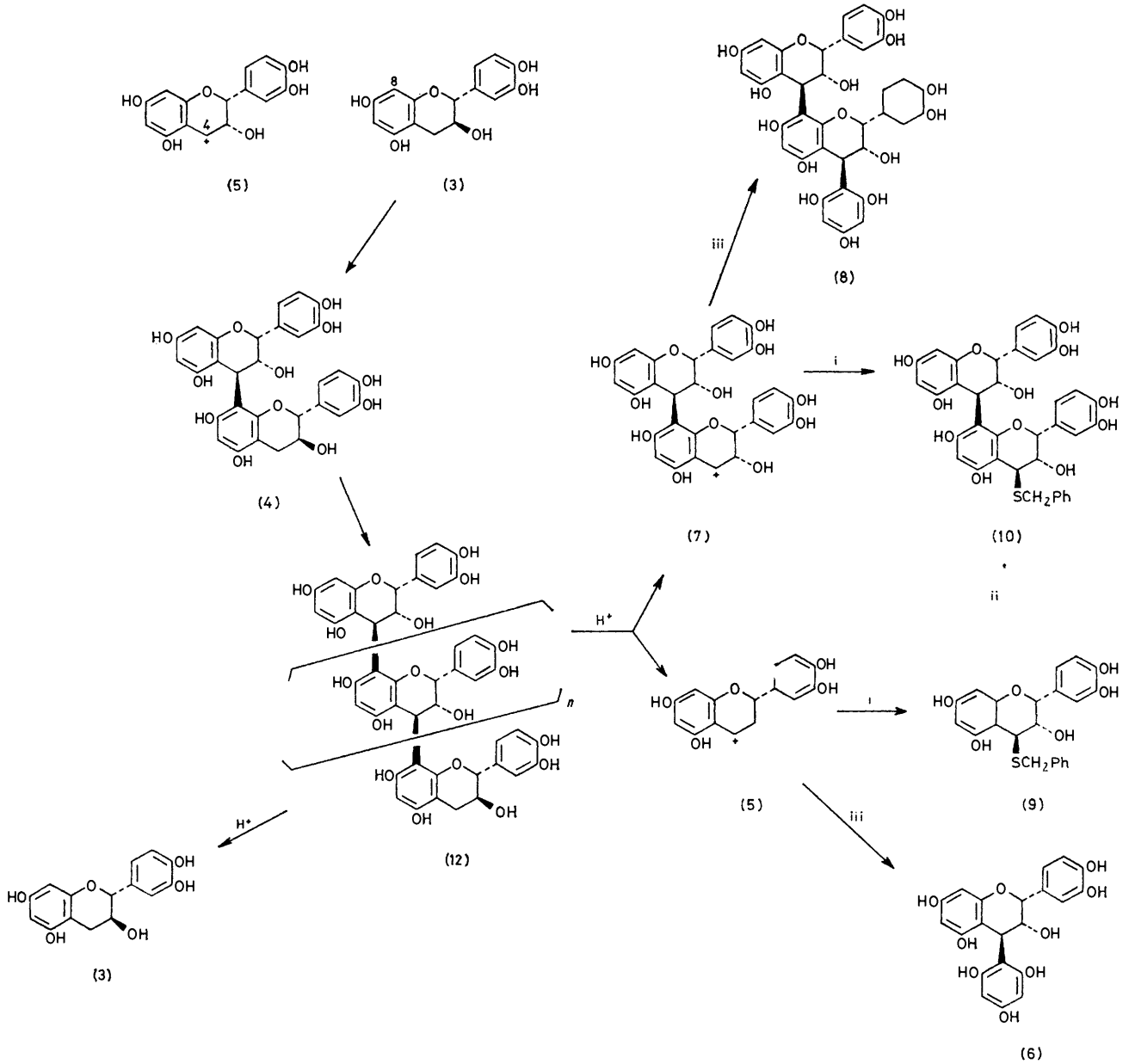
(1) R = OH
(2) R = H

condensed tannins which gave cyanidin on acidic hydrolysis.[5]

Methanolic extracts of ' very high tannin ' sorghums were analysed initially by paper chromatography and these showed one of the typical procyanidin fingerprints noted in earlier work on the plant proanthrocyanidins.[6,7]

[1] Part 4, D. Jacques, C. T. Opie, L. J. Porter, and E. Haslam, *J.C.S. Perkin I*, 1977, 1637.
[2] K. H. Daiber, *J. Sci. Food Agric.*, 1975, **26**, 1399.
[3] H. A. Stafford, *Plant Physiol.*, 1965, **40**, 130.
[4] E. C. Bate-Smith, *J. Food Sci.*, 1969, **34**, 203; *Phytochemistry*, 1969, **8**, 1803.
[5] D. H. Strumeyer and M. J. Malin, *Biochim. Biophys. Acta.*, 1969, **184**, 643; *J. Agric. Food Chem.*, 1975, **23**, 909.
[6] R. S. Thompson, D. Jacques, E. Haslam, and R. J. N. Tanner, *J.C.S. Perkin I*, 1972, 1387.
[7] E. Haslam, *Phytochemistry*, 1977, **16**, 1625.

The flavan-3-ol (+)-catechin (3) and the procyanidin dimer B-1 (4) were identified along with various luteolinidin and apigenidin glycosides. Subsequently large-scale extraction of sorghum cultivars gave extracts in these mono- and di-meric flavan-3-ol species appear to decline rapidly in concentration to leave the polymeric procyanidin as the principal and in many cases the sole procyanidin in the seed coat.

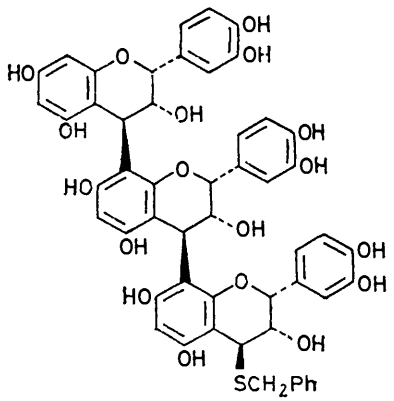


Biosynthesis and degradation of sorghum procyanidin polymer; reagents: i, $PhCH_2SH$; ii, $PhCH_2SH$—H^+; iii, phloroglucinol

which only polymeric procyanidins were present and in which the dimer (4) and (+)-catechin (3) were detected in only trace amounts. Later work revealed some of the possible reasons for this apparent change in the spectrum of phenols present in different cultivars. The sorghum grain is formed initially in a sheath and at the etiolated stage no procyanidins can be detected. As chlorophyll develops in the seed coat there is an apparently rapid synthesis of polyphenols and both (4) and (3) are present. However as the seed ripens (to a red-brown appearance) the polymeric procyanidin (up to *ca.* 5% of the grain) was isolated after chromatography of sorghum extracts on Sephadex LH-20 and gave analytical figures corresponding to a polytetrahydroxyflavan-3-ol structure (12). It contained, when pure, no sulphur or nitrogen. Treatment of the polymer with hydrochloric acid in ethanol at 60 °C gave cyanidin; it was subsequently therefore degraded by two acid-catalysed procedures.[6,8]

[8] A. C. Fletcher, L. J. Porter, E. Haslam, and R. K. Gupta, *J.C.S. Perkin I*, 1977, 1628.

Treatment with acid in the presence of phloroglucinol [8] gave as the major product the adduct (6), and (+)-catechin (3). A minor product was tentatively identified as the phenol (8). Treatment of the polymer with acid in the presence of toluene-α-thiol [6] similarly gave as identifiable products (+)-catechin, and the two thioethers (9) and (10). All these products were characterised by ^{1}H n.m.r. analysis, by the preparation of acetate and methyl ether derivatives, and, in the case of the thioether (10), by further degradation with toluene-α-thiol to yield (9). A minor product in the toluene-α-thiol degradation was tentatively presumed to be the trimeric species (11). These products of acid degrad-



(11)

ation result from the random acid-catalysed fission of the interflavan bonds in the polymer and capture of the appropriate carbocations, such as (5) and (7), by phloroglucinol or by the thiol reagent. In later work the reaction using toluene-α-thiol was pursued to completion using an excess of toluene-α-thiol and long reaction times, and in these instances the only recoverable products were (+)-catechin (3) and the thioether (9). This reaction was subsequently utilised as a basis for the estimation of the molecular weight of the procyanidin polymer. Thus the measured ratio of (+)-catechin (3) (chain termination unit) and the thioether (9) (chain extension unit) obtained from a typical degradation was 1 : 5—6, and this gives an average chain length of 6—7 flavan-3-ol units in the polymer (12; $n = 4$ or 5) (average molecular weight 1 700—2 000).

On the basis of previous work [1,7] and the observations outlined above the polymeric procyanidin in sorghum cultivars may be presumed [9] to be formed from (+)-catechin (3) and the carbocation (5) in a multiple condensation process to give a polymeric structure of the general form (12) in which the interflavan bonds are formed predominantly but not exclusively between C-4 and C-8 of the various flavan units.

EXPERIMENTAL

Mass spectra were obtained with A.E.I. MS 9 and MS 12 instruments. ^{1}H N.m.r. spectra were obtained at 60, 100, and 220 MHz and ^{13}C n.m.r. spectra at 25.15 MHz. O.r.d. and c.d. measurements were made using a Thorn–Bendix instrument. Two-dimensional paper chromatographic analysis (ascending) was carried out at 20 ± 2 °C on Whatman no. 2 paper (27.5 cm²) in the systems (A) 6% acetic acid (v/v) and (B) butan-2-ol–acetic acid–water (14 : 1 : 5 v/v). Procyanidins (+)-catechin, and (−)-epicatechin, and related compounds were detected by u.v. illumination and by the spray reagents (i) vanillin (200 mg) and toluene-*p*-sulphonic acid (100 mg) in methanol (40 ml), with heating at 80 °C for 5 min (the compounds appeared as orange-red to rose-pink spots) and (ii) iron(III) chloride–potassium hexacyanoferrate(III) (freshly prepared 0.2% solutions containing a trace of potassium permanganate). With the latter spray, after successive washes with 2N-hydrochloric acid and finally water, readily oxidisable phenols are revealed as Prussian Blue spots; the paper chromatograms when dry are permanently stable to light. Cyanidin was determined by measurement of its absorption at 535 nm in methanol containing hydrochloric acid and by chromatography on MN-300 cellulose-precoated plastic sheets using Forestal solvent [acetic acid–water–concentrated hydrochloric acid (30 : 10 : 3 v/v)] and ascending elution. Cyanidin (R_F 0.49) was detected as a rose-red spot and luteolinidin (R_F 0.61) as an orange spot, with $\lambda_{max.}$ (methanol–hydrochloric acid) 493 nm.

Preliminary Analytical Investigations.—Whole seeds (Sorghum N.K. 300; 20 g) were macerated in ethanol (50 ml) in a high-speed macerator for 10 min. The red-pink solution was filtered free of debris and carbohydrate material. (A sample acidified with hydrochloric acid showed $\lambda_{max.}$ *ca.* 500 nm.) The solution was evaporated at 30 °C and the residue triturated with ethanol (5 ml); the solution was then centrifuged to remove further polysaccharides. A sample was chromatographed in solvent systems (A) and (B) and the presence of (+)-catechin [R_F (A) 0.47, R_F (B) 0.51] and procyanidin B-1 [R_F (A) 0.51, R_F (B) 0.30] was demonstrated by co-chromatography with authentic samples. Several compounds, pink-orange in visible light, were provisionally identified on paper chromatograms as luteolinidin glycosides.

A sample (5 ml) of the ethanolic extract was acidified with 10N-hydrochloric acid (0.5 ml) and heated at 60 °C for ¾ h. The deep red solution showed $\lambda_{max.}$ 545 nm and chromatography showed it to contain cyanidin as the principal pigment.

Isolation of Polymeric Procyanidin from Sorghum.—Ripe sorghum (N.K. 300 harvested after *ca.* 120 days; 2 000 g) was ground to a fine powder in the dry state. The powder was extracted by stirring with methanol (3 000 ml) for 8—12 h at room temperature. Debris was filtered off and the procedure repeated a further two times. The combined methanolic filtrates (9 000 ml) were evaporated to *ca.* 900 ml and then extracted by light petroleum (b.p. 60—80 °C) (3 × 900 ml) to remove fats and residual chlorophyll. Finally the residual methanolic solution was evaporated to a gum and triturated with ethanol (100 ml). The ethanolic suspension was added to a column of Sephadex LH-20 (50 × 8 cm; in ethanol). The column was eluted with ethanol (1 000 ml) and then with methanol (2 000 ml). The methanolic eluate was evaporated to yield the polymeric procyanidin (' tannin ') as a brown granular powder (15.0 g, 0.75%). The polymeric procyanidin was used

[9] E. Haslam, C. T. Opie, and L. J. Porter, *Phytochemistry* 1977, **16**, 99.

for chemical analysis and degradation without further purification. A small quantity was purified by dissolution in hot water. The aqueous solution was filtered and evaporated, and the concentrated solution deposited the polymer as a buff powder. This was dried at 100 °C and 0.01 mmHg for 48 h [Found: C, 60.3; H, 5.1. $(C_{15}H_{14}O_6)_n$ requires C, 62.0; H, 4.8%; $(C_{15}H_{14}O_6 \cdot \frac{1}{2}H_2O)_n$ requires C, 60.4; H, 4.7%]. No S or N was found. The polymer was immobile in system (B) on paper chromatographic analysis but migrated as a streak (R_F 0.0—0.3) in system (A).

Reaction of Polymeric Procyanidin with Phloroglucinol.[8]—Polymeric procyanidin (10 g) and phloroglucinol (15 g) were dissolved in dioxan–water (1 : 1 v/v; 150 ml) and the acidity was adjusted to 0.5M with concentrated hydrochloric acid. The solution was maintained at 20 ± 2 °C for 48 h and then poured into water (400 ml). It was then extracted with ethyl acetate (6 × 250 ml). The extracts were dried (Na_2SO_4) and evaporated, and the residue was dissolved in ethanol (20 ml) and applied to a column of Sephadex LH-20 (40 × 8 cm) in ethanol. The column was eluted with ethanol and fractions (20 ml) were collected and analysed by paper chromatography.

Fractions 0—110 gave phloroglucinol and (+)-catechin (see below).

Fractions 146—205 gave (2R,3R,4R)-4-(2,4,6-*trihydroxyphenyl*)*flavan*-3,3′,4′,5,7-*pentaol* (6), which after rechromatography in acetone on Sephadex LH-20 was obtained as an off-white amorphous powder (2.4 g) (Found: C, 57.8; H, 4.6. $C_{21}H_{18}O_9 \cdot H_2O$ requires C, 58.3; H, 4.6%), $[\alpha]_{578}^{20}$ +106.3° (*c* 0.56 in MeOH); R_F (A) 0.56, R_F (B) 0.52. The octa-acetate was prepared in pyridine with acetic anhydride and crystallised from ethanol as small white plates, m.p. 148—150°, mixed m.p. 147—149° with an authentic sample [8] (Found: C, 59.0; H, 4.5. Calc. for $C_{37}H_{34}O_{17}$: C, 59.2; H, 4.5%); $M^{+\cdot}$ 750; $[\alpha]_{578}^{20}$ +93.6° (*c* 0.22 in $CHCl_3$) The compound had ^{1}H n.m.r. characteristics identical with those of an authentic sample. The heptamethyl [prepared by treatment with ethereal diazomethane followed by preparative t.l.c. (ethyl acetate–hexane, 7 : 3)] was obtained as a white granular solid from ethyl acetate–hexane, m.p. and mixed m.p. with an authentic sample [8] 102—104° (Found: C, 65.6; H, 6.3. Calc. for $C_{28}H_{32}O_9$: C, 65.6; H, 6.3%), $M^{+\cdot}$ 512.

Fractions 265—370 yielded the *phenol* (8) as an off-white powder (0.8 g) which could not be crystallised (Found: C, 61.0; H, 4.8. $C_{36}H_{30}O_{15}$ requires C, 61.5; H, 4.3%), $[\alpha]_{578}^{20}$ +129.5° (*c* 0.4 in MeOH), R_F (A) 0.48, R_F (B) 0.39.

Fractions 0—110 were dissolved in a small quantity of water and the phloroglucinol was separated by crystallisation. The solution was evaporated to dryness at 30 °C and the residue was dissolved in ethanol (10 ml) and chromatographed in ethanol on a column of Sephadex LH-20 (20 × 3 cm); 10 ml fractions were collected. Fractions 8—15 after removal of the ethanol and crystallisation from water gave (+)-catechin (200 mg) as needles, m.p. and mixed m.p. 177° (Found: C, 62.1; H, 5.1. Calc. for $C_{15}H_{14}O_6$: C, 62.1; H, 4.8%), $[\alpha]_{578}^{20}$ +17.8° (*c* 2.0 in EtOH); R_F (A) 0.47, R_F (B) 0.51. Tetra-*O*-methylcatechin, prepared with diazomethane, had m.p. and mixed m.p. 144—145° (Found: C, 65.8; H, 6.7. Calc. for $C_{19}H_{22}O_6$: C, 65.9; H, 6.7%), $[\alpha]_{578}^{20}$ −14.3° (*c* 1.5 in $CHCl_3$). Penta-*O*-acetylcatechin, prepared in acetic anhydride–pyridine, had m.p. and mixed m.p. 131—132° (Found: C, 60.2; H, 4.9. Calc. for $C_{25}H_{24}O_{11}$: C, 60.0; H, 4.8%); $[\alpha]_{578}^{20}$ +39.7°.

Reaction of the Polymeric Procyanidin with Toluene-α-thiol.[6]—Polymeric procyanidin (3.0 g) was suspended in ethanol (20 ml) containing toluene-α-thiol (6 ml) and acetic acid (3 ml) and the mixture was heated under reflux in nitrogen for 50 h. The solvent was removed *in vacuo* and the residual oil was chromatographed on Sephadex LH-20 (40 × 5 cm) using chloroform–ethanol (4 : 1 v/v) as eluant. Fractions (300, each 20 ml) were collected and grouped on the basis of paper chromatographic analysis in system (B).

Fractions 36—56 gave (+)-catechin, which was crystallised from water to give needles (150 mg), m.p. and mixed m.p. 177°, identified by ^{1}H n.m.r. analysis and by conversion into the octa-acetate and the heptamethyl ether as described above.

Fractions 65—85 gave the *thioether* (10) as an off-white amorphous powder (425 mg) which was further purified using chromatography on Sephadex LH-20 with acetone as eluant (Found: C, 62.9; H, 5.0; S, 4.2. $C_{37}H_{32}O_{12}S$ requires C, 63.4; H, 4.6; S, 4.6%); $[\alpha]_{578}^{20}$ +64.1 (*c* 0.53 in EtOH). The *acetate* was prepared by treatment with acetic anhydride and pyridine and was isolated after t.l.c. on silica [acetone–benzene (1 : 4 v/v) as solvent] and finally crystallisation from ethanol, as plates, m.p. 122—123° (Found: C. 61.2; H, 4.8; S, 2.9. $C_{57}H_{52}O_{22}S$ requires C, 61.1; H, 4.7; S, 2.85%); $[\alpha]_{578}^{20}$ +34.4 (*c* 0.27 in $CHCl_3$). The thioether (10) was treated with toluene-α-thiol (0.04 ml) and acetic acid (0.02 ml) in ethanol (2 ml) under reflux and with a stream of nitrogen passing through. The mixture was analysed in systems (A) and (B) at hourly intervals, and after 10 h the thioether had been completely degraded to (2*R*,3*S*,4*S*)-4-(benzylthio)flavan-3,3′,4′,5,7-pentaol, R_F (A) 0.35, R_F (B) 0.79.

Fractions 160—210 yielded a product tentatively identified as the thioether (7) (100 mg). The compound when degraded with further toluene-α-thiol and acetic acid in ethanol gave products which could be identified as the thioethers (9) and (10). The mixture finally gave only the thioether (9). Elution of the column finally with methanol gave polymeric material which could not be characterised (500 mg).

Fractions 15—35 gave a mixture of products (950 mg) which was rechromatographed in chloroform–ethanol (4 : 1 v/v) on Sephadex LH-20. Fractions were again collected and combined on the basis of paper chromatography in systems (A) and (B).

Fractions 11—22 in the rechromatography gave (2*R*,3*S*,4*S*)-4-(benzylthio)flavan-3,3′,4′,5,7-pentaol (9) (780 mg), obtained as a pale buff powder after chromatography on Sephadex LH-20 in acetone and freeze-drying from t-butyl alcohol; $[\alpha]_{578}^{20}$ −21.1° (*c* 1.2 in EtOH); R_F (A) 0.35, R_F (B) 0.79. Treatment of the thioether (2 mg) with 5N-hydrochloric acid in ethanol at 60 °C gave cyanidin. Treatment of the thioether (2 mg) in ethanol (5 ml) with Raney nickel (3 ml ethanolic slurry) for 2 h gave (−)-epicatechin, identified by paper chromatography.

The thioether tetramethyl ether, prepared with diazomethane in methanol (3 ×) and purified by t.l.c. (silica; chloroform–methanol), crystallised from methanol–water as needles, m.p. and mixed m.p.[6] 57—58° (Found: C, 66.4; H, 5.8; S, 6.7. Calc. for $C_{26}H_{28}O_6S$: C, 66.7; H, 6.0; S, 6.8%); $M^{+\cdot}$ 468; $[\alpha]_{578}^{20}$ −10.8° (*c* 0.8 in $CHCl_3$).

The thioether penta-acetate, prepared in pyridine–acetic anhydride and purified by t.l.c. (methanol–chloroform, 1 : 200 v/v), crystallised from methanol as needles, m.p. 125—126° (Found: C, 61.4; H, 5.1; S, 5.2. $C_{32}H_{30}O_{11}S$

requires C, 61.7; H, 4.8; S, 5.1%); $M^{+\cdot}$ 522; $[\alpha]_{578}^{20}$ −37.3° (*c* 0.7 in $CHCl_3$).

Number Average Molecular Weight Determination of Polymeric Procyanidin.—The degradation with toluene-α-thiol [6] was used as a basis for the number average molecular weight determination. Polymeric procyanidin (1.0 g) in ethanol (30 ml) containing toluene-α-thiol (2.0 ml) and acetic acid (1.0 ml) was refluxed under a nitrogen stream for 24 h. Further toluene-α-thiol (1 ml) and acetic acid (1 ml) were then added and the reaction continued for 125 h. The reaction mixture was then worked up as described previously and after careful chromatography the amounts of (+)-catechin (100 mg) and (2*R*,3*S*,4*S*)-4-(benzylthio)flavan-3,3′,4′,5,7-pentaol (850 mg) were accurately determined. On the basis of this result the number average molecular weight of the polymeric procyanidin is 1 700—2 000.

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