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A PROANTHOCYANIDIN FROM AVOCADO SEED*

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Abstract—A proanthocyanin has been isolated from avocado seed. The compound resembles those that have been isolated from other sources in its ready hydrolysis into catechin and the products resulting from a polyhydroxyflavan-3,4-diol, but differs from these in the manner in which the two C_{15}-units are joined. Other proanthocyanins have been assigned structures in which the two flavan-derived units are joined by ether linkages. The analytical and spectral data presented in this paper lead to the conclusion that the avocado proanthocyanin consists of two flavan-derived units joined by a carbon–carbon linkage.

INTRODUCTION

AMONG the polyphenolic compounds of higher plants are found members of a large class of substances that have long been referred to as "leucoanthocyanins".[1] These are compounds that fall into several distinct groups. One of these consists of a number of polyhydroxyflavan-3,4-diols, characteristically present in woods and barks, and convertible by heating with acids into flavylium salts which possess hydroxylation patterns different from those of the common anthocyanins. Another consists of the flavan-3,4-diols that are convertible into the common anthocyanins pelargonidin, cyanidin and delphinidin. A third includes a group of compounds which appear to consist of two flavan derivatives coupled to form a 30-carbon-atom compound which can be hydrolyzed with acid to yield a flavan-3-ol (a "catechin") and an anthocyanidin, usually cyanidin. The fourth group, related to the third, includes an ill-defined array of substances that appear to be polymeric in nature, and which can be decomposed by acid treatment with the production of anthocyanidins.[2–4]

The chemistry and stereochemistry of the "monomeric", or 15-carbon-atom, leucoanthocyanidins of the first class has been the subject of intensive study during the past several years.[1] A number of flavan-3,4-diols of the common anthocyanidin types are also known[5–9] but this group needs further study. The polymeric compounds, broadly referred to as tannins, appear to be responsible for the astringency of many plant materials.[4] Those that have been studied with the greatest care are related to the first group of flavan-3,4-diols.[10] Because of the complexity of these polymeric substances it has not yet been found possible to describe their

* Contribution No. 1764 from the Department of Chemistry of the University of California, Los Angeles.

[1] J. W. CLARK-LEWIS, in *Chemistry of Flavonoid Compounds* (Edited by T. A. GEISSMAN), p. 217, Pergamon Press, Oxford (1962).
[2] D. G. ROUX and S. R. EVELYN, *Biochem. J.* **69**, 530 (1958).
[3] E. C. BATE-SMITH and T. SWAIN, *Chem. & Ind. (London)* 377 (1953).
[4] E. C. BATE-SMITH, *Food* **23**, 124 (1954).
[5] A. K. GANGULY, T. R. SESHADRI and P. SUBRAMANIAN, *Tetrahedron* **3**, 225 (1958).
[6] K. R. LAUMAS and T. R. SESHADRI, *J. Sci. Ind. Res. (India)* **17B**, 44, 167 (1958).
[7] D. E. HATHWAY, *Biochem. J.* **70**, 34 (1958).
[8] G. R. NAGARAJAN and T. R. SESHADRI, *J. Sci. Ind. Res. (India)* **20B**, 615 (1961).
[9] A. K. GANGULY and T. R. SESHADRI, *J. Sci. Ind. Res. (India)* **17B**, 168 (1958).
[10] D. G. ROUX and S. R. EVELYN, *Biochem. J.* **70**, 344 (1958).

structures in detail, although certain reasonable conjectures have been advanced to account for the manner in which the C_{25}-units are combined.

The third group of compounds referred to above, of which the presently known examples are C_{30} compounds formed by the joining together of two flavan units, is relatively little known. Only a few well-characterized compounds of this group are known, and most of the structural assignments that have been made rest upon insecure grounds.[11-14] The importance of this group of oligomers is considerable, for an elucidation of their structures may provide evidence regarding the manner in which polyhydroxyflavan derivatives could polymerize to form the many astringent flavonoid tannins of nature.

The term "leucoanthocyanin", which has long been applied to compounds of all of the types mentioned, is an improper name, as Freudenberg has emphasized.[15] The term "proanthocyanin" is preferable; it has no explicit structural implication, and refers only to the fact that these compounds yield anthocyanidins by treatment with acids.

The present study deals with a new member of this class of dimeric compounds, isolated from the seed of the avocado (*Persea gratissima* Gaertn.). The structural evidence to be described in the sequel provides a basis for the conclusion that this proanthocyanin consists of a flavan-3-ol and a flavan-3,4-diol joined by a carbon–carbon bond.

RESULTS

Avocado seeds are rich in a complex mixture of polyphenolic compounds, ranging from the simple (+)-catechin and (−)-epicatechin to highly polymeric substances. Paper chromatograms of the total extract of the seed disclose a range of vanillin-reacting constituents in a nearly continuous streak from the origin to R_f zones typical of the catechins. The components are readily separable into three groups: the ether-soluble catechins; an ethyl acetate-extractable mixture of what are probably oligomers of moderate size; and the water-soluble but non-extractable polymers.

Isolation of the proanthocyanin. The ethyl acetate-soluble fraction of an extract of the peeled seeds discloses a number of components on a paper chromatogram, including a prominent spot at an R_f value somewhat lower than those of (+)-catechin and (−)-epicatechin. In early experiments this substance was isolated by a lengthy procedure involving column chromatography on polyamide, with the use of buffered water–methanol mixtures for elution. The course of the separation was followed by the "leuco-reaction",[16] and by paper chromatography. It was necessary to perform the column chromatography in subdued light, but even with the exercise of great care decomposition occurred on the column, and catechins appeared in eluates from materials that had been free of them when placed on the column, and consequently losses were high. It was later discovered that effective purification could be effected by a process of repeated fractional precipitation by addition of hexane to an ethyl acetate solution of the extractives.

The compound (hereinafter called the *dimer*) was not crystalline. It was astringent, and formed a white powdery solid which gradually turned a pale pink color on keeping. It gave a single, somewhat elongated spot on a paper chromatogram, the appearance of which sug-

[11] W. G. C. FORSYTH and J. B. ROBERTS, *Biochem. J.* **74**, 374 (1960).
[12] K. FREUDENBERG and K. WEINGES, *Tetrahedron Letters* 267 (1961).
[13] K. FREUDENBERG and K. WIENGES, *Angew. Chem.* **74**, 182 (1962)
[14] K. WEINGES, *Chem. Ber.* **94**, 3032 (1961).
[15] K. FREUDENBERG and K. WEINGES, *Tetrahedron* **8**, 336 (1960).
[16] W. PIGMAN, E. ANDERSON, R. FISCHER, M. A. BUCHANAN and B. L. BROWNING, TAPPI **36**, 4 (1953).

gested that the substance consisted of two compounds with substantially the same R_f value However, the conclusions regarding those features of the structure that are of special interest are not effected by this fact.

The *dimer* gave analytical figures which (with the inclusion of a molecule of water, as has been found necessary in the case of comparable compounds [14, 17]) correspond to a composition resulting from the coupling of a tetrahydroxyflavan-3,4-diol with a tetrahydroxyflavan-3-ol (a catechin) by the loss of the elements of water. Its i.r. spectrum showed no unusual features; it was very similar to that of (+)-catechin, as might be expected from the assigned structure.

Acetylation of the dimer confirmed the composition given above and in addition showed that ten acetyl groups were introduced. The i.r. spectrum of the acetate showed the absence of a hydroxyl group but had no useful features besides the expected absorptions of the acetyl groups.

Methylation of the dimer was carried out in several ways. Diazomethane failed to methylate it completely, even upon repeated treatment. Methylation by preliminary treatment with

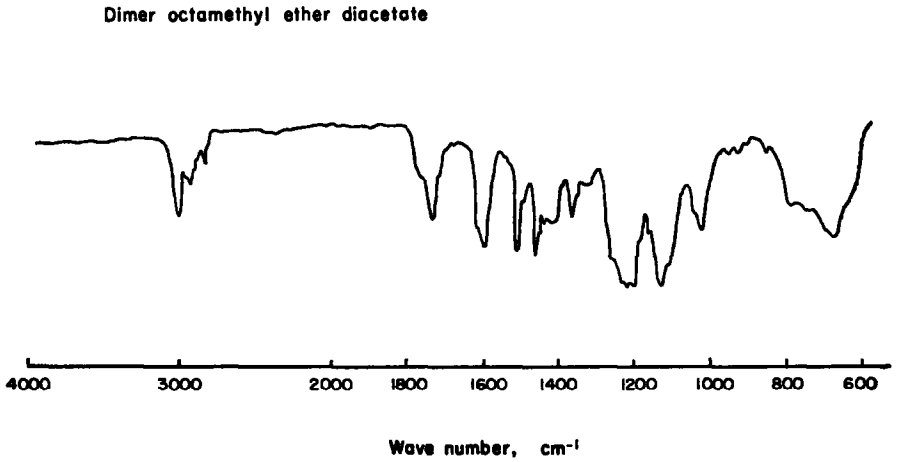


FIG. 1. INFRA-RED SPECTRUM OF PROANTHOCYANIN OCTAMETHYL ETHER DIACETATE, IN $CHCl_3$.

diazomethane, followed by dimethyl sulfate in acetone in the presence of potassium carbonate, or by dimethyl sulfate and potassium hydroxide, gave the expected octamethyl ether. The i.r. spectrum of this ether showed the presence of hydroxyl groups.

Acetylation of the octamethyl ether yielded a compound which is clearly a diacetate. Although the analytical figures show a slightly higher acetyl content than expected, the i.r. spectrum (Fig. 1) showed the absence of hydroxyl group absorption and the NMR spectrum (described below) shows the required number of acetyl group protons.

Hydrolysis of the Proanthocyanin (Dimer)

Vigorous hydrolysis of the dimer with strong acid (conc. HCl in ethanol or 1-butanol) produced the expected deep, bright wine-red color. The pigment was isolated by solvent partition and examination by paper chromatography showed it was cyanidin, along with, as is always the case in such treatment of proanthocyanins, other pigments of undetermined nature.

[17] A. K. GANGULY and T. R. SESHADRI, *Tetrahedron* 6, 21 (1959).

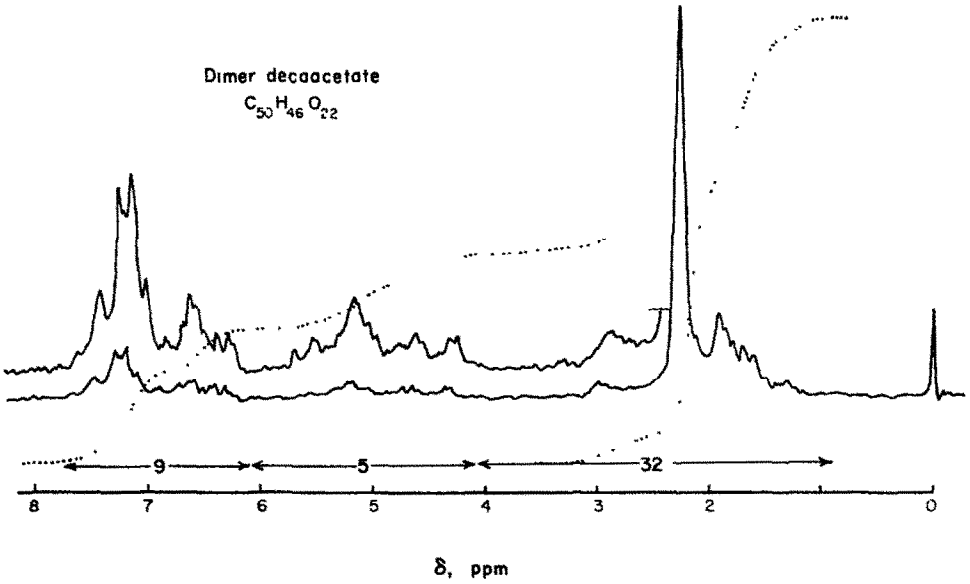


FIG. 2. NMR SPECTRUM OF PROANTHOCYANIN DECAACETATE, IN $CDCl_3$ VS. TETRAMETHYLSILANE AS INTERNAL STANDARD.

Figures show number of protons between indicated points.

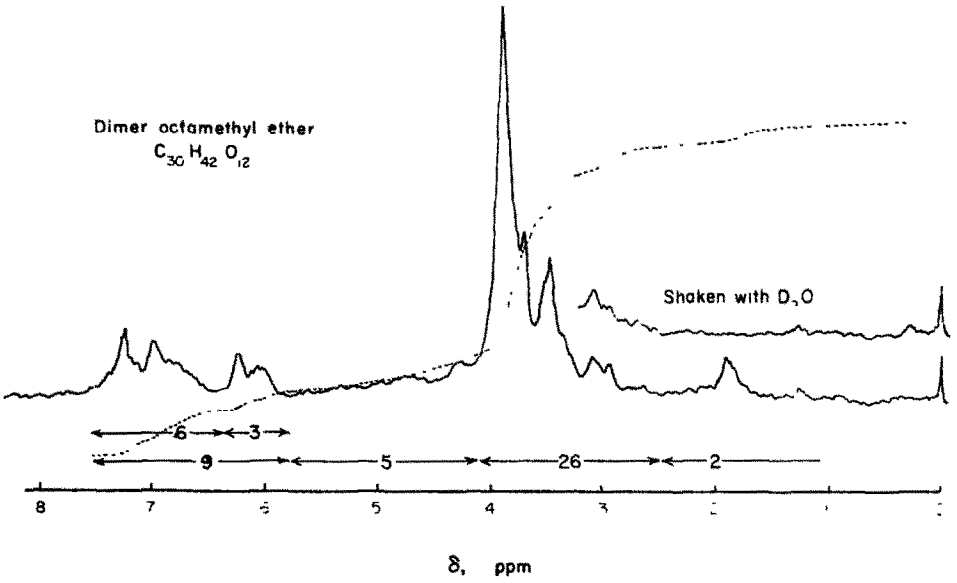


FIG. 3. NMR SPECTRUM OF PROANTHOCYANIN OCTAMETHYL ETHER, IN $CDCl_3$ VS. TETRAMETHYLSILANE AS INTERNAL STANDARD.

Figures show number of protons between indicated points.

In dilute (0·05–0·10 N) hydrochloric acid at 100° no appreciable color developed, but paper chromatography showed that the compound slowly hydrolyzes with the formation of a mixture of (+)-catechin and (−)-epicatechin. In addition, a zone of low-R_f, probably polymeric, material appears and increases in intensity with time. It is clear that the dimer is being cleaved into catechin and another component that condenses into polymeric products. Treatment of (+)-catechin under the same conditions gave no indication that polymeric condensation products are formed.

The ease with which this hydrolysis occurs shows that the linkage between the catechin unit and that which gives rise to cyanidin is very labile to acid. Of the various ways in which the two units can combine, an acetal or benzyl ether linkage would possess the acid lability

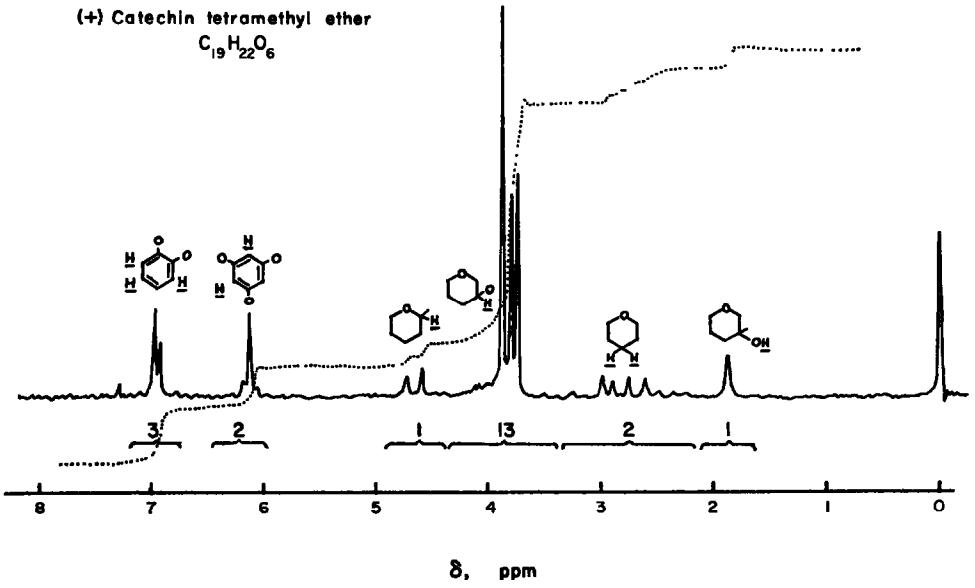


FIG. 4. NMR SPECTRUM OF (+)-CATECHIN TETRAMETHYL ETHER, IN $CDCl_3$ VS. TETRAMETHYLSILANE AS INTERNAL STANDARD.
Assignments of structural features and proton integration are shown.

required to account for the sensitivity of the dimer to acid. Such ether-linked structures have been proposed for proanthocyanins from other plants.[11–13] Another possibility that has already been offered as a conjecture,[14,18,19] is that the two units are linked by an acid-labile carbon–carbon bond.

It will be clear that certain of these possibilities can be dismissed by an examination of the data already presented. Others can be considered in the light of evidence obtained from the nuclear magnetic resonance (NMR) spectra of suitable derivatives of the dimer.

The NMR spectra of the dimer decaacetate and octamethyl ether are shown in Figs. 2 and 3. The overall proton integrations of these and that of the octamethyl ether diacetate are in complete agreement with the molecular formulas given below. The most significant feature

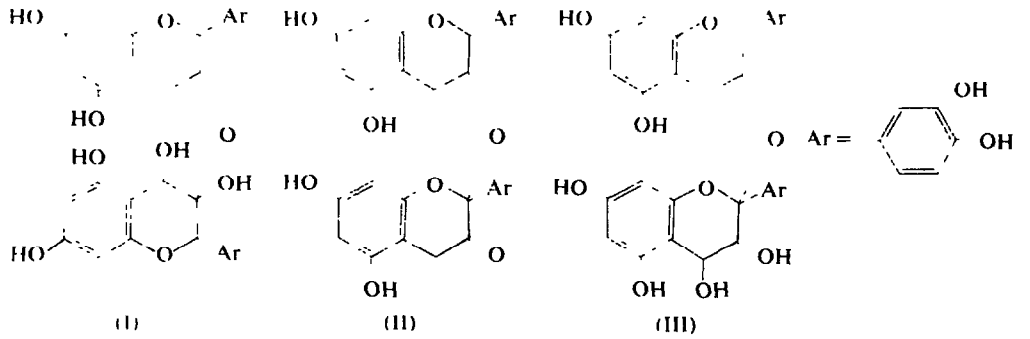
[18] K. FREUDENBERG and K. WEINGES, *Chem. & Ind. (London)* 486 (1959).
[19] H. L. HERGERT, in *Chemistry of Flavonoid Compounds* (Edited by T. A. GEISSMAN), p. 572, Pergamon Press, Oxford (1962).

of these spectra is in the region 6·0–8·0 ppm in which the signals for the aromatic protons are seen. There are two types of aromatic protons present in compounds such as catechin: the 2',3',6'-protons of the B ring, and the 6,8-protons of the A ring. The latter, which are flanked by oxygen atoms, are found upfield from those of the B ring, the separation being clearly recognizable.[20] As an example of this, the NMR spectrum of catechin tetramethyl ether (Fig. 4) shows the two A-ring protons at 6·15 ppm, and the three B-ring protons at 7·0 ppm. In each of the NMR spectra of the derivatives of the dimer (Figs. 2 and 3) there are clearly discernable two separated regions of aromatic proton absorption. These do not occur as sharply defined sets of signals as do those in catechin tetramethyl ether: nevertheless, it is easy to distinguish those of the A rings at about 6·1 ppm from those of the B rings at about 7 ppm. Proton integration of these two regions shows that the ratio B-ring: A-ring protons is 6:3 in each case. In short, there is one less proton in the A-ring region than there would be if the two phloroglucinol (A) rings and the catechol (B) rings were as in the C_{15}-"monomers".

The conclusion from this is that one of the A rings carries a substituent that has replaced one of the aromatic ring hydrogen atoms in the dimer.

DISCUSSION

Dimeric proanthocyanins have been isolated by Forsyth and Roberts (I)[11] and by Freudenberg and Weignes (II, III).[12,13] The structures that have been proposed for these compounds, all of which resemble the avocado dimer in their hydrolysis to cyanidin and epicatechin, are the following:

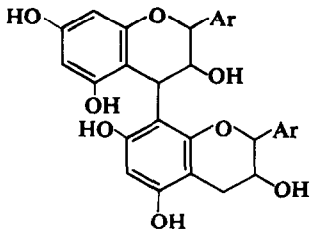


It is clear from the analytical data and from the i.r. spectrum of the avocado dimer that it cannot be identical with II, which possesses only eight hydroxyl groups and contains a carbonyl group. Structures I and III contain ten hydroxyl groups, and potential cyanidin and catechin units, but its possession of six B-ring- and four A-ring-protons would be revealed in the NMR spectrum as discussed above.

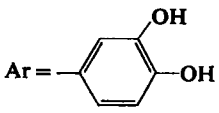
Our proposal that the structure of the avocado dimer is IV is in complete agreement with all of our experimental findings, and this structure is consistent with the mechanistic requirements for its formation and acid cleavage:*

[20] See also T. J. TATTERHAM and R. J. HIGHET, *Austr. J. Chem.* **17**, 428 (1964).

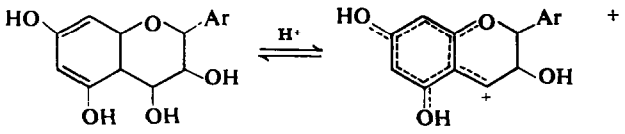
* The selection of the 8-position, rather than the 6-position, for the bond to the catechin unit is arbitrary and is still not proved. Experiments are in progress to establish this point



(IV)

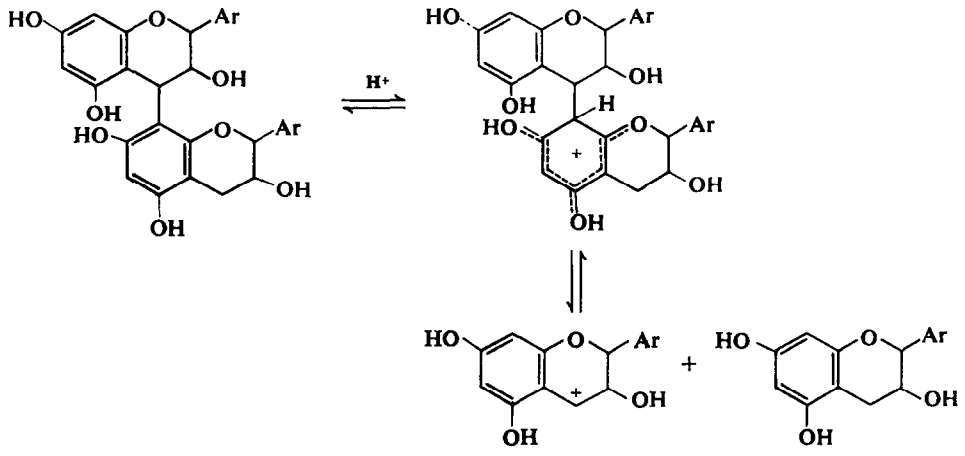


The mechanistic feasibility of an acid-catalyzed condensation[21] between a flavan-3,4-diol and a catechin is clear. The extremely reactive 4-position in a 3,4-diol such as V is evident when it is observed that carbonium ion (VI) formation at this position is enhanced by resonance stabilization by the three phloroglucinol oxygen atoms. Electrophilic substitution of



(V) (VI)

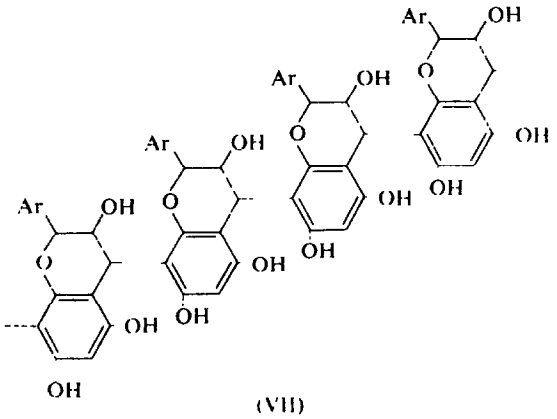
this fragment into the highly active phloroglucinol ring of another like molecule would be expected to be ready, and reversible. The reversibility of this reaction under acidic conditions can be represented by the following:



(VI)

It is also apparent that self-condensation of VI and the flavan-3,4-diol could occur by the same mechanism, and can lead to polymerization to a compound that can be represented as VII:

[21] See K. FREUDENBERG and K. WEINGES in *Chemistry of Flavonoid Compounds* (Edited by T. A. GEISSMAN), p. 197, Pergamon Press, Oxford (1962).



(VII)

It is possible that VII represents the polymeric proanthocyanins that occur widely in nature.[1,2,10] When the condensation of VI occurs with a catechin unit, the product, which then would lack the 4-hydroxyl group through which further reaction can occur, would be relatively stable and would not undergo further condensation of the kind under discussion. Thus, a flavan-3,4-diol, through VI, would be expected to undergo self-condensation (or condensation with other flavan-3,4-diols) to polymeric tannins, or condensation with catechins to dimeric proanthocyanins.

EXPERIMENTAL

Isolation of the dimer. Freshly peeled avocado seeds (5·6 kg) were macerated with methanol in a blendor and re-extracted twice more. The combined extract (22 l.) was evaporated under reduced pressure in a cyclone evaporator and the concentrate (2 l.) kept in the refrigerator for 15 hr. A copious precipitate of inositol (100 g) was removed and the filtrate extracted with ethyl ether (20 × 400 ml) to remove fatty materials and the bulk of the catechins. The aqueous solution was then extracted with ethyl acetate (14 × 400 ml) to yield a total of 46·3 g of extractables. The fractions were kept separately and chromatographic examination showed that 1 to 5 were complex mixtures containing large amounts of the catechins. Extracts 6–9 showed three main constituents and in later fractions these were still present, some of them of lower intensity

Fractions 6–14 were combined and redissolved in ethyl acetate (350 ml); of the 15·7 g of crude material 4·9 g would not redissolve. The filtered solution was treated with hexane in portions, each precipitate being collected (Table 1). Precipitates 2–6 were combined and redissolved in ethyl acetate in which they were completely soluble. Fractional precipitation with hexane was repeated twice, the first and last fractions always being discarded. The product

TABLE 1. FRACTIONAL PRECIPITATION OF ETHYL ACETATE SOLUBLE FRACTION

	Number						
	1	2	3	4	5	6	7
Hexane added (ml)	10	25	50	50	50	50	100
Precipitate (g)	0·25	1·7	2·5	2·5	1·2	1·0	0·4

finally obtained was homogeneous as shown by paper and thin-layer chromatography (silica gel-G; ethyl acetate:chloroform:acetic acid, 20:10:1), with an R_f 0·57 on paper with the developing solvent 1-butanol:acetic acid:water, 10:3·7:6·3. A vanillin-toluenesulfonic acid spray was used to reveal the proanthocyanin on the paper. The final yield of material giving a single but slightly elongated spot was 4·0 g.

Earlier experiments in which polyamide column chromatography was used gave material which proved to be identical with the substance isolated as described above. The much more tedious column purification procedure was wasteful, and will not be described here.

The proanthocyanin is a white, amorphous solid that has no definite melting point (decomposes at about 250°). (Found: C, 60·69, 60·53; H, 4·58, 4·69. Calc. for $C_{30}H_{26}O_{12}\cdot H_2O$: C, 60·40; H, 4·73%) It has a strongly astringent taste. When heated with concentrated hydrochloric acid in 1-butanol it gives a clear wine-red color with a magenta cast, quite unlike the darker, brownish-red colors that are obtained when most crude polyphenolic materials ("tannins") are treated in this way.

Controlled hydrolysis. A 20 mg sample of the proanthocyanin was dissolved in 2 ml of 0·05 N or 0·1 N hydrochloric acid and heated in a boiling water bath. Paper chromatograms were run with (1) a mixture of (+)-catechin and (−)-epicatechin; (2) the initial proanthocyanin hydrolyis solution before heating was started; and (3) samples removed from the hydrolysis solution after varying times of heating. The appearance of catechin and epicatechin, and of polymeric materials at lower R_f values was clearly evident.

Proanthocyanin deca-acetate. A solution of 0·52 g of the well-dried proanthocyanin in 2·5 ml of dry pyridine and 10 ml of acetic anhydride was allowed to stand for 24 hr at 35°. The solution was poured into water and the mixture allowed to stand for 5 hr at 4°. The solid product was collected, and washed with water and dried. The crude acetate (0·79 g) was dissolved in ethanol and the solution diluted with an equal volume of water containing a few drops of acetic acid. The pure compound that separated (0·56 g) appeared to be crystalline but melted over the range of 155–165°. A thin-layer chromatogram showed a single spot. (Found (specimen from column-purified material): C, 60·24, 60·29; H, 4·64, 4·69; acetyl, 42·2, 41·5, 41·4, 41.7. (Specimen prepared by solvent fractionation): C, 59·94, 59·63; H. 4·78, 4·77; acetyl 43·6, 42·4, 42.7. $C_{50}H_{46}O_{22}$ required: C, 60·12; H, 4·64; acetyl, 43·0%)*.

Proanthocyanin octamethyl ether. A solution of 0·80 g of the proanthocyanin in 8 ml of methanol was treated in portions with a freshly distilled ethereal solution of diazomethane until the evolution of nitrogen no longer occurred. Since the addition of ether caused the partial precipitation of the unmethylated proanthocyanin, the process was repeated after removal of the solvent and addition of fresh portions of methanol and diazomethane. The final reaction mixture was kept for several hours at −10° and then evaporated to dryness under reduced pressure. The product at this stage was not completely methylated as shown by a low methoxyl content and the presence of strong hydroxyl absorption in the i.r. Two procedures for further methylation were followed.

In one of these, the crude diazomethane-methylated product was treated with freshly distilled dimethyl sulfate in dry acetone in the presence of anhydrous potassium carbonate. The product was isolated by evaporation of the filtered solution and purified by precipitation from ethanolic solution by dilution with water.

* Acetyl groups determined by a modification of the Kuhn–Roth method for the estimation of C-linked methyl groups. In control runs, the following results were obtained: (+)-catechin pentaacetate, calc. 43·1% acetyl; found, 42·2, 41·5, 42·5; glucose pentaacetate, calc. 55·1% acetyl; found, 54·6, 55·0; methyl glucuronate tetraacetate, calc. 45·8% acetyl; found, 45·7.

In another experiment the crude diazomethane-methylated product was dissolved in 20 ml of methanol and to this solution was added 25 ml of 50% aqueous potassium hydroxide, followed by the equivalent quantity of dimethyl sulfate in small portions. The reaction mixture was kept alkaline at all times. The mixture was finally heated on the steam bath for 30 min and cooled, and the light-brown solid product collected and dissolved in ethyl acetate and the solution washed with water, dried and evaporated. A solution of the residue in ethanol was diluted with water and the precipitated material reprecipitated in the same way. The final product was a white solid that was not crystalline but was readily filterable. It melted over a range of 160–164°. (Found (methylation with diazomethane only): C, 64·40; H, 5·82; OCH_3, 27·04, 26·80. (Successive methylation with diazomethane and then dimethyl sulfate, acetone, K_2CO_3): C, 65·91; H, 6·25; OCH_3, 34·38, 34·85. (Successive methylation with diazomethane and then dimethyl sulfate, potassium hydroxide): OCH_3, 35·33, 35·24. $C_{30}H_{18}O_4(OCH_3)_8$ required: C, 66·07; H, 6·12; OCH_3, 35·95%.)

Proanthocyanin octamethyl ether diacetate. The octamethyl ether was acetylated with acetic anhydride and pyridine in the manner described above for the preparation of the deca-acetate. The product, precipitated from ethanol solution by dilution with water melted over the range 157–162° and was apparently not crystalline. Its i.r. spectrum showed the absence of absorption in the hydroxyl region, and the analytical values showed that two acetyl groups were present. (Found: acetyl, 12·3, 13·1, 12·4, 13·2, 13·0. $C_{40}H_{44}O_{13}$ (octamethyl ether monoacetate) required: acetyl 6·8%. $C_{42}H_{46}O_{14}$ (octamethyl ether diacetate) required: acetyl, 11·1%. $C_{43}H_{46}O_{15}$ (heptamethyl ether triacetate) required: acetyl, 16·1%.)

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