

# Identification of the Main Oligomeric Proanthocyanidin

## Occurring in Apples

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The main proanthocyanidin found in Waldhoefer apples has been isolated from pressed juice by a one-step procedure using column chromatography. The properties and the mass, IR, and NMR spectra of its derivatives obtained by methylation and acetylation were examined and compared with the data of the corresponding derivatives of the four isomeric

Type B proanthocyanidins ( $C_{30}H_{26}O_{12}$ ) reportedly occurring in fruits. The apple proanthocyanidin was identical to the compound  $B_2$  isolated from cola nuts, hawthorn berries (*crataegus oxyacantha*), and cocoa beans, and hence is a dimer of two (-)epicatechin units linked together by a  $C_4-C_8$  bond.

An earlier paper (Van Buren *et al.*, 1966) described the isolation and the chemical characteristics of the main oligomeric proanthocyanidin extractable from Waldhoefer apples, an astringent-tasting juice variety. Although properties and analytical data accumulated for the free substance and its methyl derivative suggested the structure of a dimeric polyphenol, the degree of polymerization as well as its true structure could not be elucidated fully.

Despite the application of modern analytical techniques, the conformations of the condensed procyanidins isolated from fruits remained uncertain because mass spectral data do not reveal differences between closely related isomers (Weinges and Freudenberg, 1965) and the NMR spectra presented so far did not show enough resolution (Geisman and Dittmar, 1965) or cannot be interpreted in an unambiguous manner (Weinges *et al.*, 1968b; Mayer *et al.*, 1966). Recently, the structure of a crystalline  $C_{30}H_{26}O_{12}$  procyanidin isolated from cola nuts has been established by synthesis, proving that the two flavanoid units are linked between  $C_4$  of the "upper" pyran ring and  $C_8$  of the "lower" phloroglucinol ring (Weinges and Perner, 1967). Among the procyanidins which give cyanidin and either (+)catechin or (-)epicatechin on acid hydrolysis, only four different compounds with the same elemental composition, called  $B_1$  and  $B_4$ , have been found in various fruits (Weinges *et al.*, 1968b). Assuming that these isomers are biogenetically formed by dehydration of two monomeric flavanoid units, the two halves of the dimeric procyanidins should be linked by a  $C_4-C_8$  bond and should have the conformation of either (+)catechin or (-)epicatechin. On the basis of these inferences, and disregarding the configuration at  $C_4$ , Weinges *et al.* (1968a) were able to establish the conformations of the four isomers by means of their 100-MHz NMR spectra.

Since the apple proanthocyanidin yielded cyanidin and (-)epicatechin upon acid hydrolysis and was believed to have the formula  $C_{30}H_{26}O_{12}$ , it would be expected to belong

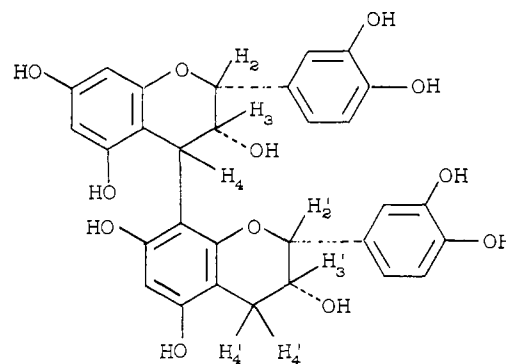


Figure 1. Structure of the main oligomeric apple procyanidin

to the procyanidins of this B-group. The chemical and spectral data presented in this paper show the identity of the apple procyanidin as the  $B_2$  isomer (Figure 1).

### EXPERIMENTAL

The apples (variety Waldhoefer) were harvested four to six weeks before ripeness and held frozen until used.

For the preparation of the juice, the apples were thawed in lukewarm water and processed with a commercial juice press. The freshly pressed juice was kept cool by addition of dry ice and filtered with suction through asbestos wool. The clarified juice was used immediately for the isolation of the polyphenols or kept under refrigeration for later use.

For the isolation of the polyphenols, a polyamide column was prepared. The polyamide powder (Woelm) was suspended in distilled water for 24 hours. The supernatant water was decanted. The polyamide was resuspended in fresh distilled water, poured into the chromatography column, and allowed to settle while the water drained from the bottom of the column. The clarified apple juice (1 liter per 15 grams of dry polyamide powder) was percolated through the column, followed by a water washing, until the effluent was tasteless (400 ml. per liter of juice). The polyphenols were eluted from the column with 95% ethanol and fractionated at the same time. The

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ethanol effluent was collected by means of an automatic fraction collector (10 ml. per fraction).

The methyl derivative of the main procyanidin was prepared as previously described (Van Buren *et al.*, 1966). The sample used for the mass spectrum was additionally purified by chromatography on a silicic acid (0.05- to 0.2-mm., Merck) column with a 4-to-1 mixture of benzene and acetone (v./v.) (Weinges *et al.*, 1968b).

Acetylations were carried out using a 1-to-1 mixture of dry pyridin and distilled acetic anhydride. The samples were dissolved in this mixture and kept at room temperature for several days with exclusion of moisture. Excess reagent was then removed under vacuum at 40° C. in a flash evaporator. The residue was dissolved in chloroform and extracted several times with ice water. The chloroform solution was dried over sodium sulfate and brought to dryness in a flash evaporator. The acetyl derivatives were purified by column chromatography on silicic acid (0.05- to 0.2-mm., Merck) using ethyl acetate for the first run and a 4-to-1 mixture of benzene and acetone (v./v.) for the second run (Weinges *et al.*, 1968b).

Thin layer chromatography of the free polyphenols was carried out as previously described (Van Buren *et al.*, 1966). The methyl and acetyl derivatives were run on silica gel (silica gel G, Merck) thin layers with the solvent systems benzene-acetone = 4 to 1 (v./v.) (Weinges *et al.*, 1968b) and chloroform-ethyl acetate = 1 to 1 (v./v.).

IR spectra were recorded using the KBr disk technique (1 mg. of sample per 300 mg. of KBr). The 100-MHz NMR spectra were obtained in deuteriochloroform. Internal standard was tetramethylsilane.

## RESULTS AND DISCUSSION

Using two-dimensional chromatography on thin layers of cellulose, the main procyanidin, previously called sub-

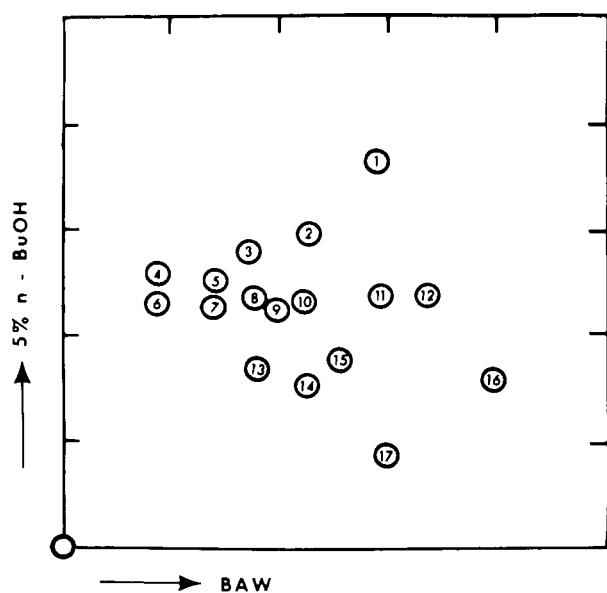


Figure 2. Cellulose thin-layer chromatogram of Waldhoefer apple polyphenols

BAW: *n*-Butanoläcetic acid-water = 30 to 5 to 11

stance No. 2 (Van Buren *et al.*, 1966) was detected in ethyl acetate extracts of juices prepared from a number of different varieties of apples and pears. Only four spots appeared on the chromatogram of the extract of freshly prepared juices of unripe Waldhoefer apples. These spots correspond to the spots numbered 1 (chlorogenic acid), 2 (main unknown procyanidin), 8 or 9 (unknown), and 15 [(-)epicatechin] in the chromatogram presented previously (Van Buren *et al.*, 1966) (Figure 2). Even in more concentrated extracts (+)catechin (spot No. 12) could not be detected, but was found in acetone extracts of the residual press cake of the Waldhoefer apples.

The main unknown procyanidin was easily isolated from the juice using chromatography on a polyamide column. Passing the clarified juice of 1 kg. of Waldhoefer apples (650 ml.) through the column (10 grams of polyamide), the polyphenols and anthocyanins were adsorbed in the top section of the column. The effluent juice, shown to contain no polyphenols, had a sweet-sour taste and did not exhibit astringency. Subsequent to washing the column with water (250 ml.), ethanol (95%) was used as eluent. The anthocyanins and most of the chlorogenic acid traveled with the ethanol front. The elution pattern of the other polyphenols is summarized in Table I.

Identification of the substance in fraction A, previously based on its chromatographic behavior (Van Buren *et al.*, 1966), was confirmed by comparison of its acetyl derivative with an authentic specimen of pentaacetyl (-)epicatechin. The melting point of the acetate which crystallized from ethanol in colorless needles was 149-50° C., and showed no melting point depression when mixed with the reference compound.

In the mass spectrum of the methyl derivative of the unknown procyanidin prepared from fraction B, the molecular ion appeared at *m/e* 690. This is in accordance with the elemental analysis of this derivative (Van Buren *et al.*, 1966) and proves its dimeric structure. Thus, the formula for the octamethyl derivative is C<sub>38</sub>H<sub>32</sub>O<sub>12</sub>. The spectrum showed characteristic peaks at *m/e* 672, 654, 511, 493, 492, 479, 477, 461, 331, 327, 299, 191, 180, 179, 167, 165, 151, and 137. This fragmentation pattern is in agreement with the suggested structure (Figure 1) because analogous or identical fragments are characteristic of this type of structure (Weinges *et al.*, 1968b; Weinges and Freudenberg, 1965).

Acetylation of fraction B gave a derivative which could

Table I. Polyphenols Recovered from the Juice of 1 Kg. of Waldhoefer Apples by Adsorption on Polyamide

Fraction	Substance No.	Vol. of Fraction, Ml. Ethanol	Weight, Mg.
A	15	40	157.3
B	2	190	159.0
C	8 or 9	600	79.4

Intermediate fractions combined		270	96.8
		Total	492.5

not be crystallized. Since precipitation with water from ethanolic solutions (Weinges *et al.*, 1968b; Geisman and Dittmar, 1965) could chromatographically be shown to give artifacts, it was precipitated from ethyl acetate with petroleum ether. The amorphous white acetate gave only one spot on thin layer chromatograms (solvent a:  $R_f = 0.46$ ; solvent b:  $R_f = 0.50$ ) and showed a melting range of 124–8° C. and a specific optical rotation of  $[\alpha]_D^{25} = 51^\circ$  (c, 2.7 acetone). The elemental analysis gave C, 59.88%, and H, 4.71%, in accordance with the formula  $C_{50}H_{46}O_{22}$  for a decaacetate of a dimeric flavanoid (calculated: C, 60.11%, and H, 4.66%). The 100-MHz NMR spectrum of this acetyl derivative confirmed the presence of 10 acetyl groups ( $\delta = 1.82$  to 2.38 p.p.m.) and nine aromatic protons, with two protons showing two sharp doublets at  $\delta = 5.98$  p.p.m. and  $\delta = 6.22$  p.p.m. clearly separated from the multiplet originating from the other seven aryl protons ( $\delta = 6.50$  to 7.40 p.p.m.). The chemical shifts and the coupling constants found for the protons of the two pyran rings are summarized in Table II.

These data were in agreement with those reported for the acetyl derivative of the  $B_2$  procyanidin which has been isolated from cola nuts, hawthorn berries (*crataegus oxyacantha*) and cocoa beans (Weinges *et al.*, 1968b). The identity of the two compounds was established by comparing the decaacetyl derivative of the apple procyanidin with an authentic sample of the acetate of the  $B_2$  procyanidin. They could not be separated by cochromatography on thin layers of silica gel, showed no change in melting behavior upon mixing, and gave identical IR spectra.

Since the structure of the  $B_2$  procyanidin has been established recently (Weinges *et al.*, 1968a; Weinges *et al.*, 1968b), the apple procyanidin should also be a dimer of two (–)epicatechin units linked by a bond between  $C_4$  of the “upper” and probably  $C_8$  of the “lower” unit (Figure 1).

The point of attachment of the “lower” unit is still uncertain, since the data obtained so far do not discriminate between positions of  $C_6$  and  $C_8$  of the phloroglucinol ring. With the fact in mind that electrophilic substitutions occur at  $C_8$  of the catechins rather than at  $C_6$  (Weinges *et al.*, 1968b; Weinges and Perner, 1966) and having proved the  $C_4$ – $C_8$  bond of the  $B_1$  procyanidin by synthesis, Weinges *et al.* (1968a) inferred the same type of bonding for all four isomeric Type B procyanidins for biogenetic reasons. Certain observations made in the present methylation studies appeared to support this view. Additional treatments of the octamethyl derivative of the apple procyanidin with diazomethane at room temperature followed by acetylation gave a product whose mass spectrum showed the expected molecular ion of the octamethyl diacetyl derivative at  $m/e$  774 and a peak of about  $2/3$  of the intensity of the molecular ion at  $m/e$  788. No ions with higher

**Table II. Chemical Shifts and Coupling Constants for the Pyran Ring Protons of the Decaacetate of the Apple Procyanidin**

Proton No.	$\delta$ , P.P.M.	$J$ , Hz.	
2	5.56	2–3	<1
3	5.17	3–4	2.0
4	4.44		
2'	4.54	2'–3'	<1
3'	5.09	3'–4' <sub>a</sub>	<1
4'	2.93	3'–4' <sub>b</sub>	3.2
		4' <sub>a</sub> –4' <sub>b</sub>	12.0

mass could be detected. The ions with the highest mass show a difference of 14 mass units, which indicates that an additional methyl group must have been introduced to the octamethyl diacetyl derivative, probably by C-methylation of an aromatic ring. The spectrum showed basically the same pattern which has been published for the octamethyl diacetyl derivatives of the Type B procyanidins (Weinges *et al.*, 1968b). But the peaks arising from fragments which contained the “upper” half of the molecule were accompanied by new peaks at higher mass numbers differing by 14 mass units (654–668, 639–653, 503–517, 477–491, 461–475, 343–357, 327–341, 299, 313, and the doubly charged ions at  $m/e$  246–253). If it is true that only the  $C_8$  positions of the catechin units are reactive enough for this kind of C-methylation, this would mean that only one such position is available, and hence that the other is involved in the bond connecting the two catechin units.

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