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STRUCTURE ELUCIDATION OF SOME PROANTHOCYANIDINS IN BARLEY BY ^{1}H 270 MHz NMR SPECTROSCOPY

by

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In the present paper the composition of proanthocyanidins isolated from barley is investigated. Separation by affinity chromatography and HPLC yields a large number of substances. Six of these have been isolated in this work. Based on the chromatographic preparation they are expected to be two dimers *D1* and *D2* and four trimeric substances *T1* – *T4*. Studies by means of ^{1}H NMR spectroscopy at 270 MHz confirmed *D1* and *D2* as being dimeric compounds, a prodelphinidin and procyanidin B_3 respectively. The spatial structure of the prodelphinidin corresponds to procyanidin B_3. The peracetates of all six compounds have also been studied by means of ^{1}H NMR spectrocopy. By comparison with data for the analogous peracetates of (+)-catechin and (–)-epicatechin and with literature data, *T1* – *T4* have been identified as trimeric proanthocyanidins and their structures have been elucidated.

1. INTRODUCTION

Proanthocyanidins, in the brewing literature normally termed anthocyanogens, constitute a group of flavonoid compounds of significant practical interest to the brewing industry since it has been shown (10) that they participate in the formation of haze in beer through reactions with proteins. The origin of most of the proanthocyanidins in beer is the malt, which contains all the proanthocyanidins of the barley (11). From

Abbreviations: HPLC = high-pressure liquid chromatography, NMR = nuclear magnetic resonance, TMS = tetramethylsilane.

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barley and malt three procyanidins and one prodelphinidin have previously been characterized (9, 14). The constitution of the three procyanidins was deduced by proving their identity to authentic species (9, 14). The prodelphinidin could not be identified in this manner since no reference substance was available, leaving open the possibilities that the dimeric prodelphinidin had either a procyanidin B_1- or a procyanidin B_3-like structure (9, 14).

In the present work we have reinvestigated the composition of the proanthocyanidin fraction of barley with the primary aim of studying the prodelphinidins.

The separation of the proanthocyanidins is accomplished by a combination of low pressure affinity chromatography (Sephadex LH-20) and high-pressure liquid chromatography (HPLC) on reversed phase. The identity and structure of the compounds prepared were ascertained by chemical methods and ^{1}H NMR spectroscopy.

The reinvestigation showed that the structure of the previously mentioned dimeric prodelphinidin (9, 14) is analogous to that of procyanidin B_3. Observations leading to the same conclusion have been published by PORTER et al. (7). Three new trimeric proanthocyanidins were isolated from barley (12).

The spectroscopic results and their interpretation are discussed in this paper resulting in proposed structures for these compounds.

2. MATERIALS AND METHODS

2.1. Isolation of proanthocyanidins from barley

The barley variety investigated was Nordal, harvest 1978 and 1979. The method of preparation of pure proanthocyanidins has been described elsewhere (12) and will only be stated briefly.

Barley kernels were decorticated in the Carlsberg Research Mill, the flour fraction being isolated for further investigations. The fine particles of this decorticated material were separated from coarse proanthocyanidin-free particles by means of bolting, hereby leaving a flour containing 8–9 $mg \cdot g^{-1}$ of proanthocyanidins, which were extracted with acetone-water (3:1) (9). An initial fractionation was carried out by low pressure chromatography on a Sephadex LH-20 column with 96% ethanol-water (3:2). Following this procedure the proanthocyanidins were fractionated mainly according to molecular weight (12). Further separation of groups of proanthocyanidins was obtained by preparative HPLC.

In this manner it was possible to separate prodelphinidins and procyanidins. The two trimeric proanthocyanidins which are at the same time prodelphinidins and procyanidins could not be separated by HPLC. The separation was accomplished by renewed fractionation on Sephadex LH-20.

For convenience in the following discussion labels have been defined for the six compounds. The labels *D* and *T* refer to the number of catechin units, whereas the numbering (e.g. *D1* and *D2*) refers to the sequence of elution by HPLC. Thus *D1* is more polar than *D2*.

2.2. Acetylation of proanthocyanidins

The proanthocyanidin (about 10 mg) was dissolved in pyridine (200 µl). The solution was treated at room temperature with acetic acid anhydride (200 µl). When deuterated acetates for spectroscopic purposes were needed $(CD_3CO)_2O$ (Merck Sharpe & Dohme Canada Limited, Montreal) has been used. After 16 hours the reaction mixture was poured on ice, whereby the acetate precipitated. The precipitate was washed with cold water until the smell of pyridine disappeared. After the washing the precipitate was dried in vacuum at room temperature at 10^{-2} torr.

2.3. Hydrolysis of proanthocyanidins

A few milligrams of proanthocyanidin were treated with 0.1 N HCl in ethanol for 15 min at 60 °C according to HASLAM et al. (13). By this procedure the lower terminal unit was split off as a flavan-3-ol. After oxydative hydrolysis in Bu^tOH – HCl (5:1) for half an hour at 100 °C the upper units are transformed into anthocyanidins.

2.4. Preparation of NMR samples

The proanthocyanidins were dissolved in deuterioacetone-d_6 (Merck, Darmstadt, purity $\geqslant$ 99.5%) and tetramethylsilane (TMS) (Merck, Darmstadt) was added as internal standard. The

solutions were placed in 5 mm o.d. sample tubes. The acetylated proanthocyanidins were dissolved in $CDCl_3$ (Merck, Darmstadt, purity $\geqslant$ 99%) and the solutions were placed in 5 mm o.d. sample tubes. The concentrations were in the range 2–5% w/v.

2.5. ^{1}H NMR spectra

The ^{1}H NMR spectra have been obtained using a BRUKER HX270S spectrometer operating in Fourier transform mode. Spectra were accumulated in 32K data points using a spectral width of 3000 Hz. The repetition time was ~5 sec and a pulsewidth of 10 μsec corresponding to 50° flip angle was used. The number of accumulated scans varied from 100 to 500 depending on concentration and experimental conditions. Decoupling experiments have been used extensively to confirm the assignments reported in the tables and figures in section 3. The spectrometer was operated at 30 °C. The chemical shift data are referenced to $CHCl_3$ (δ = 7.37 ppm) for $CDCl_3$ solutions and to TMS for solutions in deuterioacetone-d_6. In order to improve resolution Gaussian lineshapes have been introduced as described by FERRIGE and LINDON (5).

2.6. Nuclear Overhauser experiment (NOE)

The proton proton NOE experiment was performed by alternating selective irradiation at the frequencies of the C_4 protons in (+)-catechin with a total saturation period of 5 sec. The reference spectrum was obtained with the decoupling frequencies outside the spectral region. The total accumulation time was 15 hours. The two spectra are swopped from disc after 16 scans in order to minimize the influence of long term effects.

2.7. Spectral interpretation

The ^{1}H NMR spectra consist in general of a large number of lines which may be assigned to partly overlapping small spin systems, since the protons in the individual rings do not interfere. As a consequence it has been possible to analyse most of the spectra by standard methods.

3. RESULTS

3.1. Chemical evaluations

According to the Sephadex LH-20 chromatogram (12), the proanthocyanidins were divided into two groups, the proposed dimers *D1* and *D2* and the proposed trimers named *T1–T4*. Some

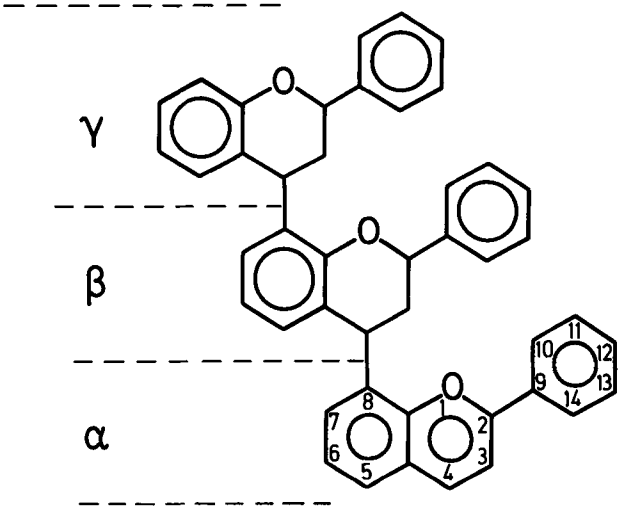

Figure 1. Numbering and labelling in proanthocyanidins straightly C_4–C_8-linked.

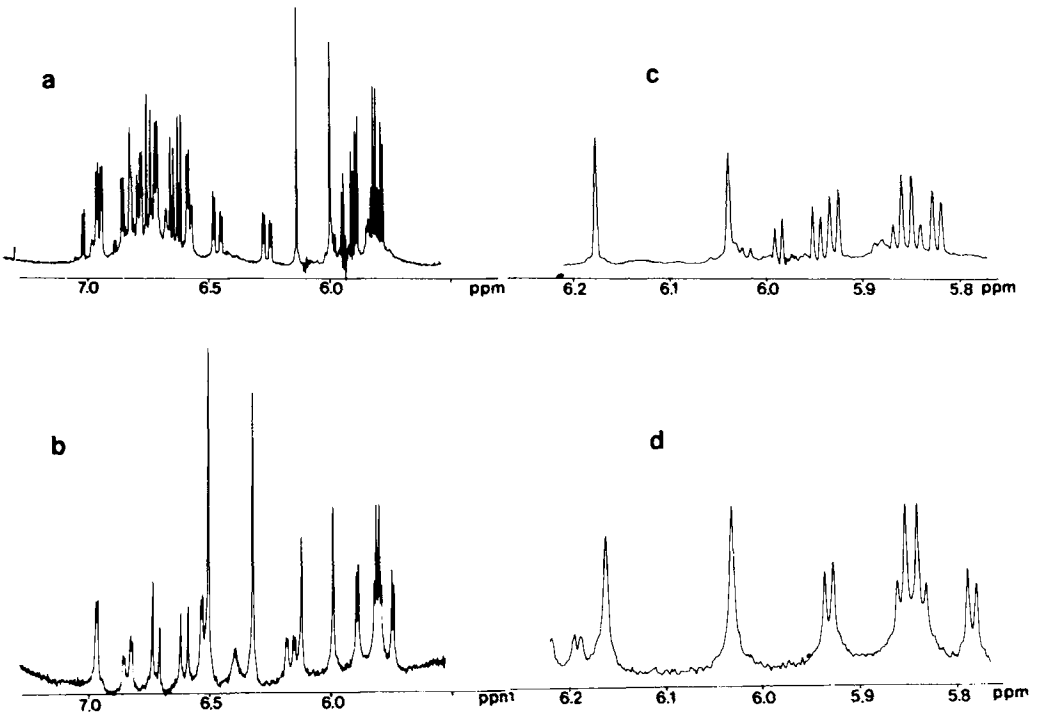

Figure 2. The 270 MHz ^{1}H spectra of the aromatic regions of two dimeric proanthocyanidins.

2a shows the spectrum of synthetic procyanidin B_3, 2b shows the spectrum of prodelphinidin B_3 isolated from barley. 2c and 2d show the high field part of 2a and 2b, respectively, on an expanded scale. The impurity observed in the spectrum of B_3 is assumed to arise from taxifolin.

of their chemical properties were as follows:

Common to all six components was the liberation of (+)-catechin on mild acid hydrolysis (2.3).

On stronger acid hydrolysis (2.3) the identified anthocyanidins were the following:

D1: Delphinidin
D2: Cyanidin
T1: Delphinidin
T2: Delphinidin and cyanidin
T3: Delphinidin and cyanidin
T4: Cyanidin

3.2. ^{1}H NMR analysis

In Figures 2a and 2b the aromatic region of the ^{1}H NMR spectrum of *D1* and *D2* is reproduced. Figure 2c shows the high field part of 2a including the H_6 and H_8 protons. The broad lines interfering with the sharp signals from the aromatic protons are due to phenolic OH. A similar interference between the pyrane ring signals and the aliphatic OH signals is also found. These effects prevent the use of integration in the identification beyond the qualitative level. The abundance of signals observed in *D1* and *D2* arise from the presence of two conformations of the molecules under the experimental conditions.

The spectra of the peracetylated compounds in $CDCl_3$ show only one dominant isomer and the absence of OH signals makes the assignment possible. In Figure 3 the chemical shifts are reported for the alicyclic parts of the ring systems in the peracetylated proanthocyanidins studied. The corresponding aromatic part of the chemical shifts is reported in Figure 4. The accuracy of the spectral analysis was better than 10^{-2} ppm. It was found, however, that concentration and temperature dependent variations of the chemical shifts exceeded this value by a factor two to five.

In order to keep track of the signals due to all the proanthocyanidins investigated in a consis-

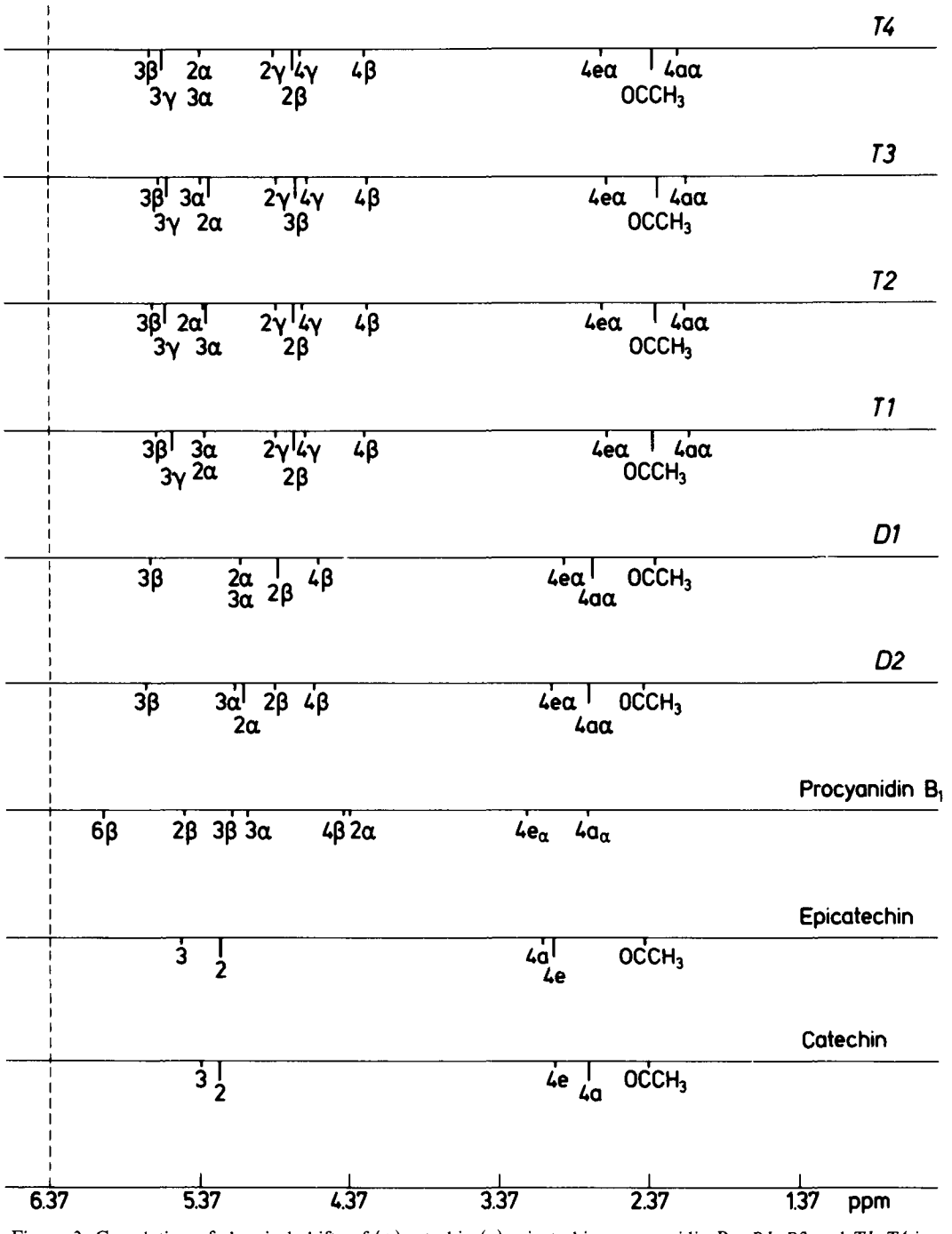

Figure 3. Correlation of chemical shifts of (+)-catechin (−)-epicatechin, procyanidin B_1, *D1–D2* and *T1–T4* in $CDCl_3$ solutions, aliphatic part.

tent way we have numbered the atoms in the monomer units from 1–14 and labelled the units α, β, γ as indicated in Figure 1.

The assignment of H_6 and H_8 in (+)-catechin is possible due to the difference in distance from H_4 to H_6 and H_8. The ring puckering motion in (+)-catechin is not well known, but a coarse estimate based on Dreiding models makes

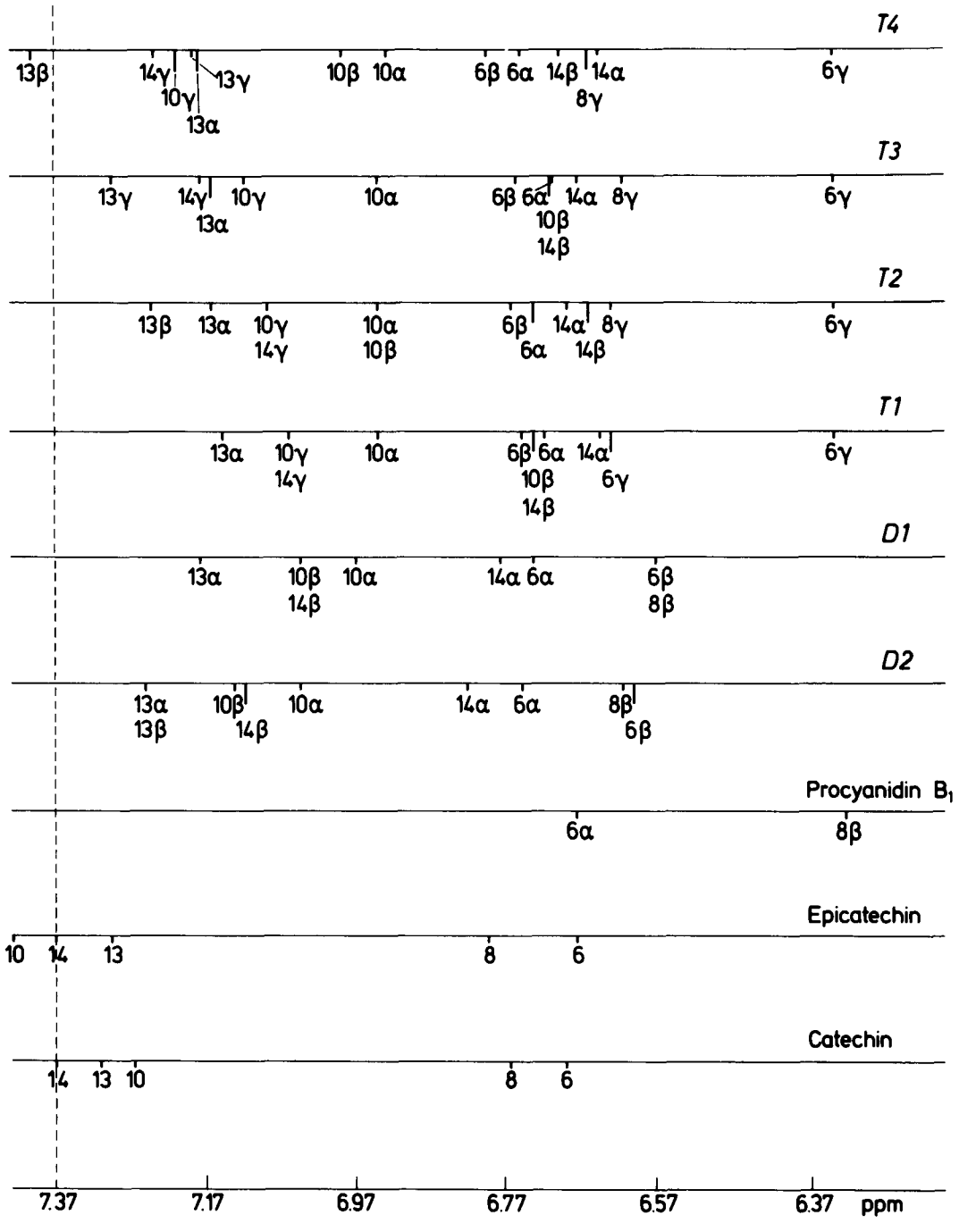

Figure 4. Correlation of chemical shifts of (+)-catechin, (−)-epicatechin, procyanidin B_1, *D1–D2* and *T1–T4* in $CDCl_3$ solutions, aromatic part.

expected NOE on H_6 about five times larger than on H_8. The NOE experiment (2.6) resulted in a NOE effect 4 times larger on H_6 than on H_8.

The measured coupling constants are reported in Table I. The main interest is focused on coupling constants in the pyrane ring. The (+)-

Table I

Spin spin coupling constants in peracetylated proanthocyanidins dissolved in $CDCl_3$, J_{ij} in Hz

i	j	B_1[a]	B_3[a]	*D1*	*D2*	*T1*	*T2*	*T3*	*T4*
2α	3α	10	–	–	–	–	–	–	–
3α	4eα	–	–	4	6.0	–	–	–	–
3α	4aα	–	–	5.5	7.5	–	–	–	–
4eα	4aα	–	–	16	16.5	17.5	17.5	17.1	16.5
10α	14α	–	–	2.2	2.0	~1	2.0	2.0	2.0
13α	14α	–	–	8.4	7.8	8.8	8.3	8.5	–
2β	3β	<1	9.5	10.5	9.2	10.2	10.1	10.0	10.2
3β	4β	2.1	9.5	9.5	9.2	9.1	8.8	8.8	9.0
6β	8β	–	–	2	2.2	–	–	–	–
10β	14β	–	–	–	–	–	1.8	–	1.8
13β	14β	–	–	–	7.8	–	8.3	–	–
2γ	3γ	–	–	–	–	10.2	10.3	10.3	10.0
3γ	4γ	–	–	–	–	8.4	8.3	8.6	8.5
6γ	8γ	–	–	–	–	2.1	2.4	2.2	2.3
10γ	14γ	–	–	–	–	–	–	2.0	–

[a] Data from ref. 15 and 16.

catechin units all give rise to axial-axial orientation of the proton pairs H_2/H_3 and H_3/H_4. The magnitude of these coupling constants have previously been reported by WEINGES et al. (16) to be approx. 10 Hz. In contrast (–)-epicatechin is characterized by very small coupling constants (16).

4. DISCUSSION

The chromatographic separations resulted in the purification of six different proanthocyanidins being present in decorticated barley flour. The retention data indicate that two dimeric (*D1* and *D2*) and four trimeric (*T1–T4*) compounds are present. The acid hydrolysis (2.3) permits the identification of the lower terminal unit (α in Figure 1) as a (+)-catechin in all six compounds. For the upper units (β, etc.) the chemical identification previously performed (12) is unable to distinguish (+)- and (–)-configurations. Thus it can only be established whether these units are catechin or gallocatechin. The polarity of six substances differ, judged on the basis of chromatography. The polarity is found to increase with the proportion of gallocatechin. The dimeric compounds are *D1*, a prodelphinidin and *D2*, a procyanidin. The four trimeric compounds include a less polar procyanidin *T4* and a strongly polar compound prodelphinidin *T1*. The remaining trimeric compounds appear to be procyanidin and prodelphinidin simultaneously. Thus, the aim of this work was to establish the configuration of the upper unit of the dimeric prodelphinidin and to elucidate the structures of the previously unknown three trimeric proanthocyanidins by ^{1}H NMR spectroscopic analyses.

From Table I and Figure 3 it is seen that in all the proanthocyanidins isolated from barley the configuration of the methylene protons in the α-unit, although shifted towards high field, is as in (+)-catechin, i.e. opposite the configuration in (–)-epicatechin. This is in full agreement with the observation that all these proanthocyanidins split off (+)-catechin on acid hydrolysis.

In Figure 2 the aromatic part of the spectra of unacetylated *D1* and *D2* are represented. The multitude of signals in both compounds correlates closely with the observation made by HASLAM et al. (13), that dimeric proanthocyanidins with a (+)-oriented β-unit in acetone exist as a mixture of two slowly interchanging conformers. In Figure 2c it is seen that two secondary structures are present in almost equal quantities (6). Since *D2* in every respect is identical with procyanidin B_3 (9, 12, 13, 16, 17), it is assumed that the procyanidin *D2* is identical with this compound.

The prodelphinidin *D1* possesses the same configuration as *D2* in the upper unit judged by

the magnitude of the coupling constants (Table I). Thus, the β-unit must have a (+)-configuration, i.e. it must be a (+)-gallocatechin unit. In Figure 2d it is seen that the secondary structures present in *D1* correlate closely with those of procyanidin B_3.

The same prodelphinidin has previously been isolated from Ribes Sanguinea (7). The reported NMR data coincide with our observations. In the assignment of the signals the locations of $H_{2\beta}$ and $H_{4\beta}$ are, however, reversed.

The mentioned multitude of signals in the aromatic region (Figure 2) is accompanied by a similar doubling of the line pattern in the aliphatic region. Furthermore the total spectrum is hampered by a multitude of OH-signals (compare with Figure 2). In a communication FEENEY (4) reports the observation of slow exchange of phenolic OH protons with water present in the acetone used as solvent. It would therefore appear promising to eliminate the OH-signals by exchange with D_2O. When this experiment was carried out it was, however, observed that the aromatic signals were diminished as well. It is assumed that this phenomenon is due to the existence of quinone tautomeric forms in proanthocyanidinic polyphenols allowing deuterium to be incorporated in the aromatic rings. In addition the free polyphenols are very sensitive to oxidation, making it very difficult to maintain the integrity of such a sample over a longer period necessary for experiments. All these disadvantages made it advantageous to use protected compounds for spectroscopic studies.

Several communications (7, 15, 16) have advocated the peracetylation adopted in this study.

Referring to Figures 2, 3 and 4 it is interesting to notice that the peracetylated compounds only display one set of ^{1}H NMR signals. HASLAM et al. (6) reported for solutions of the decaacetate of procyanidin B_3 at 30 °C in dimethylsulfoxide-d_6 and in nitrobenzene-d_5 two sets of signals indicating the presence of two conformers in unequal quantities. Temperature studies yielded an estimate for the interconversion barrier ΔG of 20 Kcal/mole. The observation of one set of signals for solutions in $CDCl_3$ therefore would be consistent with one dominating conformer being present. In this respect our data for procyanidin B_3 concur within experimental accuracy with the data reported by WEINGES et al. (16). As all the trimeric proanthocyanidins investigated in this study show a similar pattern, it is suggested that $CDCl_3$ interferes with their molecular configurations yielding only one observable rotamer. Choosing $CDCl_3$ as solvent makes it relatively easy to interpret the spectra and to correlate the signals to distinct protons.

In Figure 3 and Figure 4 the data for procyanidin B_3 *(D2)* and the dimeric prodelphinidin *(D1)* are represented on the same scale as those of (+)-catechin, (−)-epicatechin and procyanidin B_1 (15, 16). The δ-values for the four trimeric compounds *(T1–T4)* are represented in the upper halves of the same figures.

Significant differences between gallocatechin-containing and catechin-containing substances are found in the aromatic region. Comparing Figure 2a and Figure 2b it is seen that the gallocatechin unit is characterized by a strong singlet arising from H_{10}/H_{14} protons (cf. Figure 1). In prodelphinidin B_3 *(D1)* the H_{10}/H_{14}-signal is situated at $\delta = 7.03$ ppm. Furthermore, when regarding the locations of such signals, it is possible to deduce the number and sequence of monomer units.

Comparing with *T1*, yielding delphinidin on acid hydrolysis, it is seen that this compound displays a singlet at 7.03 ppm. This is taken as evidence of a gallocatechin unit as an upper terminal unit (γ-unit). An additional singlet is found at $\delta = 6.73$ ppm. This signal is identified as a gallocatechin in the β-unit. These signals and the integral of the spectrum define *T1* as a trimer.

The singlet indicating a gallocatechin unit in the γ-position is observed in *T2*. This information, combined with the integral defines *T2* as a trimer containing only one gallocatechin unit. In *T3* a similar argument leads to the conclusion that this compound contains gallocatechin as the β-unit. Such an argumentation also excludes the presence of gallocatechin units in *T4*.

The information obtained by hydrolysis of *T1–T4* do not exclude the presence of (−)-epicatechin in these compounds. However, the location of the 6β-signal in procyanidin B_1 (15, 16) excludes the presence of (−)-epicatechin as a unit in the four trimers investigated. The aliphatic signals arising from the pyrane rings are very similar in all four compounds (cf.

Figure 3). This indicates that all these proanthocyanidins possess the same configuration in all the pyrane rings. The measured coupling constants (Table I) are consistent with 2,3-trans-configurations of the protons, i.e. (+)-catechin configuration of the pyrane ring in all cases.

When the sequences of units have been fixed, the question of linkage has to be discussed, i.e. whether the interflavan linkages connect C_4 and C_6 carbon atoms or C_4 and C_8 atoms.

HASLAM et al. (6, 13) have observed that among the two isomer procyanidins B_3 and B_6 the former, i.e. the C_4–C_8 interflavan bonded compound, is the most widespread in nature, the latter representing only 15–20% of the total amount. The proof of B_3 as being C_4–C_8 linked was presented by WEINGES et al. (17). Accordingly most authors have claimed investigated proanthocyanidins to exhibit C_4–C_8 linked structure.

When investigating more complex systems, i.e. dimeric and trimeric proanthocyanidins, several conformers are possible for the free forms, but in the case of peracetylated compounds, under the conditions employed in this study, only one rotamer was seen for each. The spectral patterns observed for *T1–T4* ensure that the isolated chromatographic fractions are homogeneous with respect to interflavan linkages. Since these compounds are the dominant trimeric proanthocyanidins in barley (12) it is assumed that in any case the linkages are C_4–C_8 as anticipated in Figure 1.

In the literature several papers have been concerned with the assignment of H_6 and H_8 in (+)-catechin. In an early paper BATTERHAM et al. (1) studied a large number of flavanols by ^{1}H NMR. Based on consideration of substituent effects they concluded that $\delta H_8 > \delta H_6$.

Lately ROUX et al. (3, 8) have synthesized pure 6-bromo-(+)-catechin and pure 8-bromo-(+)-catechin and used them to prepare a number of known 6- or 8-substituted catechins. The observation of the ^{1}H NMR spectra of such derivatives, lead to the suggestion of an empirical rule whereby $\delta H_8 - \delta H_6 \sim 0.1$ ppm (2). In no case overlapping of these signal ranges was observed.

In the previous discussion it has already been mentioned that proanthocyanidins may exist in two long-lived secondary structures (6). This was seen in Figures 2c and 2d, where $H_{6\alpha}$ gives rise to two signals with a separation of 0.13 ppm. It may therefore be concluded that the influence of the secondary structure on the chemical shift of $H_{6\alpha}$ is larger than the chemical shift difference used by ROUX (2) to differentiate between C_4/C_6 and C_4/C_8 isomers. Unless the assumption of equal secondary structure is invoked, it seems that the question of this isomerism in proanthocyanidins is still open for discussion.

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