Pergamon

PII: S0031-9422(96)00210-5

Phytochemistry, Vol. 43, No. 1, pp. 265–272, 1996

0031-9422/96 $15.00 + 0.00

UNAMBIGUOUS ASSIGNMENTS FOR FREE DIMERIC PROANTHOCYANIDIN PHENOLS FROM 2D NMR

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(*Received* 18 *December* 1995)

Key Word Index—2D NMR; ^{1}H NMR; ^{13}C NMR; proanthocyanidins; procyanidin dimers; free phenols.

Abstract—Characterization of proanthocyanidin oligomers proceeds commonly through investigation of NMR data of their peracetates or methyl ether acetates, in conjunction with FAB-mass spectrometry and circular dichroism. Since such an approach is unsuitable in bioassay-guided isolations, we applied two-dimensional NMR techniques for the identification of dimeric proanthocyanidins. This afforded not only a powerful probe for distinction between the different procyanidin isomers, but also allowed full assignments, even for both major rotameric forms, whenever present, without the need for derivatisation. Moreover, discrimination between the crucial 6- and 8-protons and carbons was achieved after addition of traces of cadmium nitrate, resulting in the separation of the broad phenolic signals into sharp singlets. As an example of the general strategy followed in the assignment and combination of data of the different spectra available, complete analysis of underivatised procyanidin B3 or catechin-(4$\alpha\rightarrow$8)-catechin is discussed for the first time. Copyright © 1996 Published by Elsevier Science Ltd

INTRODUCTION

The naturally widely distributed proanthocyanidins have been the subject of extensive structural investigation during the past 25 years. However, their identification and NMR characterization still remains a difficult topic in natural product chemistry. FAB-mass spectrometry provides the M_r of the unknown proanthocyanidin and, thus, gives an indication of the number of hydroxyl groups of the composing units, the degree of polymerization and the presence of doubly-linked entities. Further structural elucidation is carried out using NMR spectroscopy, generally in combination with circular dichroism. The major problems that remain to be solved are the characterization of chain-extender and terminal units, and their sequence, including their absolute configuration, the definition of the hydroxylation pattern in A- and B-rings, the assessment of bonding positions and the stereochemistry of the interflavan linkage.

The interpretation and subsequent deduction of spectral parameters from NMR spectra of proanthocyanidins is hampered by several spectroscopic limitations. First of all, rotational isomerism is an important complicating factor. Several researchers (e.g. [1]) describe a solvent-dependent manifestation of the hindered rotation about the interflavan bond. Molecular models of dimeric procyanidins suggest that there are two energetically preferred conformations, that rationalize the duplication of NMR resonances for dimers with (+)-catechin upper units in acetone-d_6. The different stereochemical situations at C-4 is responsible for the fact that, although rotatory restriction is present both in dimers with (+)-catechin and (−)-epicatechin upper units, the energy barriers differ significantly. Dimers with (−)-epicatechin upper units exhibit only a broadening of signals in the same solvent.

Therefore, derivatization as peracetates or methyl ether acetates was considered necessary for unambiguous NMR analysis. Moreover, these derivatives allow a simplified chromatographic separation [2, 3]. At ambient temperatures however, the methyl ether acetates and peracetates show complex ^{1}H NMR spectra due to broadening and duplication of spectral lines. Temperature elevation to 100° in $CDCl_3$ overcomes the energy barrier and allows free rotation about the interflavan bond for methyl ether acetate derivatives of procyanidins with 2,3-*trans*- and 3,4-*trans*-configurations. For methyl ether acetates of procyanidins with 2,3-*trans*-3,4-*cis*-entities, an even higher coalescence

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temperature is required. This urged Kolodziej to use $(CDCl_2)_2$ as solvent, permitting temperatures up to 170° while similar chemical shifts are retained [4]. Nevertheless, complete sharpening of resonances of 3,4-*cis*-procyanidins was still incomplete.

Moreover, the structural determination of proanthocyanidins was traditionally a combination of spectroscopic data and thiolytic degradation. The observation made by Hemingway and coworkers that thiolytic cleavage is faster for $4\rightarrow8$ than for $4\rightarrow6$ dimers has extensively been used as the main structural [5]. However, in 1992, McGraw *et al.* reported on a side-reaction occurring during thiolysis of condensed tannins, thus questioning all structures established through such a degradative method [6].

However, since derivatization and thiol cleavage are unsuitable for bioassay-guided strategy in the isolation of natural products, we attempted the application of two-dimensional NMR techniques for the structural elucidation of proanthocyanidins. Careful combination of correlations available from different 2D-techniques not only afford a tool for distinguishing between the different isomers but also provide a full assignment of all NMR signals, for both dominating rotamers when present. Furthermore, it is worth noting that, although in the literature the assignment of H-6/H-8 or C-6/C-8 is most often obtained by assuming analogy with data of the corresponding monomers (or 4-substituted flavan-3-ols) or of the extensively investigated derivatives (peracetates or methyl ether acetates), even for the monomers (+)-catechin and (−)-epicatechin, and for flavonoids in general, contradictory chemical shifts are reported for C-6/C-8 and H-6/H-8. In *Carbon-13 NMR of Flavonoids* Agrawal also concluded that common errors are the reversing of assignments for C-6 and C-8, the latter generally being the upfield resonance [7]. Taking into account that, for example, for catechin-$(4\alpha\rightarrow8)$-catechin or procyanidin B3, where NMR spectra taken in acetone-d_6 exhibit duplication of all signals due to rotational isomerism, six C-6 and C-8 resonances are situated in a range of 1.38 ppm (upper and lower units of both rotamers) and four H-6/H-8 doublets are found over 0.11 ppm, application without further proof of such comparative methods for assignments is not justified.

The ambiguity of the 6/8 assignments for flavonoids in the literature has already been noted by several authors. Van Loo *et al.* reinvestigated the structural assignment for apigenin by the application of 2D-heteronuclear correlation techniques in combination with selectively ^{1}H-decoupled ^{13}C NMR spectra, since they also noticed an uncertain and even contradictory 6/8 assignment in the literature [8].

Kiehlmann and Tracey analysed catechin derivatives with bromine substitution at C-6 or C-8 and substitution at oxygen by methyl, acetyl and/or hydrogen. They found the difference between H-6 and H-8 chemical shifts an unreliable criterion for the distinction between 8- and 6-bromo isomers, because of the dependence on the nature of the solvent and of the oxygen substituent [9]. The empirical NMR parameters, derived by Hundt and Roux [10] for 6- and 8-substituted tetra-*O*-methyl-catechins falling into two non-overlapping regions for H-8 (6.32–6.47 ppm) and H-6 (6.10–6.22 ppm) resonance signals in $CDCl_3$ appear critical for diflavanoids, in cases where observed chemical shift differences are usually small and the relative chemical shift parameters do not fall well inside the ranges determined for monoflavonoids. Kiehlmann and Tracey determined H-6 and H-8 frequencies for catechin *via* selective decoupling of 5-OH and 7-OH protons after addition of a trace cadmium nitrate to the substrate solution in acetone. Cadmium nitrate reduces the exchange-rate of the OH-protons to such an extent that phenolic protons appears as sharp lines instead of the broad OH-peaks. Consequently, assignments published previously (H-6: 5.88 ppm H-8: 6.02 ppm) had to be reversed [11].

Two dimensional NMR techniques have already been used in the structural elucidation of flavonoids and proanthocyanidins. Shen and coworkers reported complete assignments of the proton and carbon signals of (+)-catechin, (−)-epicatechin, dihydroquercetin and dihydromyricetin by means of HETCOR, COLOC and HMBC experiments and, subsequently, reassigned the proton signals of H-6 and H-8 [12].

Balas and Vercauteren published an NMR study on the discrimination between the $4\rightarrow8$ (B3) and $4\rightarrow6$ (B6) coupled peracetylated catechin dimers. They claimed that analysis of the 'native' dimers (or free phenolic dimers) did not allow complete assignment, considering that crucial correlations fall into a crowded area. Since polymethylated derivatives showed similar spectra, they considered acetylation to be necessary [13]. However, as will be discussed here, signals of parent, free phenolic procyanidins can be fully assigned by careful analysis of 2D-spectra with adequate resolution. This includes complete characterization of all signals from the two conformers present. Since these researchers apparently also had their doubts about the assignments based solely on chemical shift differences or degradation studies, they applied a combination of COSY, HMBC and HMQC to characterize unequivocally the location of the interflavan linkage. The key correlations as a proof for the $4\rightarrow8$ linkage are the H_{4u}–C_{8a} and H_{2t}–C_{8a} signals in the HMBC spectrum of procyanidin B3. In the case of procyanadin B6, the H_{4t}–C_{5t} and H_{4u}–C_{5t} correlations prove the $4\rightarrow6$ bond.

We report here the application of 2D homo- and heteronuclear NMR techniques for the complete assignment of free phenolic proanthocyanidins. As an example of the general strategy followed in the assignment and combination of data of the different spectra available, the analysis of underivatized procyanidin B3 or catechin-$(4\alpha\rightarrow8)$-catechin is discussed for the first time.

RESULTS AND DISCUSSION

In the following rationale limited use is made of specific chemical shift data in order to demonstrate and stress the enormous capability of the various 2D-NMR

techniques in the unambiguous interpretation of proanthocyanidin spectra.

In close analogy to the procedure described by Fonknechten *et al.* [14], condensation of reduced (+)-taxifolin with (+)-catechin afforded a series of 2,3-*trans*-procyanidins. The products were separated in the free phenolic form on Sephadex LH-20 into oligomeric categories, the biflavanoids being resolved individually under these conditions. Initial identity of the major product as procyanidin B3 was derived from the synthesis of procyanidins with precursors of known absolute configuration [15, 16], when taken in conjunction with the parent ion of *m/z* 577 in the negative ion FAB-mass spectrum and the strong negative Cotton effect in the diagnostic wave-length region of the CD spectrum, which was indicative of a 4α-flavanyl substituent. [14, 15]. Since chemical shifts cited in the literature were either of peracetylated or methyl ether acetylated products whose spectra were recorded in $CDCl_3$ at elevated temperatures, direct assignment by extrapolation was not possible. Nevertheless, investigation of the NMR data provided ample evidence to come to unambiguous identification as described below. Unambiguous proof of the configuration at the interflavan bond was provided by circular dichroism.

In the ^{13}C APT spectrum (100 MHz), the 12 upfield signals from δ 28.64 to 83.68 were due to the aliphatic carbons 2, 3 and 4 in upper and lower units of the major and minor rotamers. The two CH_2-carbons (positive in the APT-spectrum) at δ 28.64 and 28.82 were readily assigned to the C4 terminal (*t*) units. Consequently, the highest field signals but two at δ 37.97 and 38.09 are due to the C-4s of the upper (u) unit. These signals provide a distinct key entry point into the two dimensional spectra. The most downfield clusters from δ 144.85 to 145.68 and from δ 154.59 to 158.47 arise from the deshielded, phenolic carbons 3′,4′ and 5,7 of each constituent flavanyl unit, respectively, with the exception of carbon 8a (*ca* δ 158). The remaining quaternary signals resonating at δ 100.55–107.65 and at δ 131.43–132.44 are then due to the carbons 1′, 4a and the 6 or 8 carbon involved in the interflavan linkage. As a consequence the aromatic CH resonances at δ 96.09–97.47, 114.97–116.07 and 119.52–120.69 are caused by the free C-6 and C-8, C-2′,C-5′ and C-6′.

In the ^1H spectrum (400 MHz) too, all the signals occurred as pairs; in crowded regions, however, they were not always adjacent to each other. There was a constant intensity ratio of *ca* 1.3:1 between twin peaks, corresponding to the existence of two conformers (rotamers) in the same ratio. For the minor isomer, an index 'min' is added after the signal assignment, Table 1).

Initially, data from ^1H–^1H COSY and long-range COSY, ^1H–^{13}C HETCOR and long-range HETCOR experiments (Table 1) were used for the structural elucidation of procyanidin B3.

The HETCOR-spectrum displayed one-bond correlations between the C-4*t* carbon of the minor rotamer at δ 28.64 and two double doublets at 2.62 (J = 8 and 16 Hz) and 2.89 ppm (J = 5.6 and 16 Hz) (H4*t* min), and between the other (major) C-4*t* carbon at δ 28.82 and the double doublets at 2.54 (J = 8 and 16.4 Hz) and 2.79 ppm (J = 5.6 and 16.4 Hz) (H-4*t*).

In the ^1H–^1H COSY spectrum below 5 ppm, two clearly distinct sets of resonances could be seen. H-4*t* at δ 2.54 correlates with H-4*t* at δ 2.79. Both are correlated with a proton multiplet at δ 3.83, which, in turn, shows a cross-peak with the doublet at δ 4.59 (J = 7.2 Hz). Therefore, the signal at 3.83 ppm is H-3*t*, whereas the doublet is H-2*t*. In a completely analogous manner, the signals at δ 2.62 and 2.89, at 4.08 (*multiplet*) and at 4.74 (*d*, J = 7.6 Hz) are attributed to the H-4*ts*, H-3*t* and H-2*t* of the minor rotamer. Consequently, the HETCOR spectrum allows assignment of the directly bonded carbons; the ^{13}C resonances at 68.10, 68.31, 82.07 and 82.89 ppm were thus assigned to C-3*t* min, C-3*t*, C-2*t* and C-2*t* min, respectively.

The C-4*u* signals at δ 37.97 and 38.09 exhibit HETCOR cross peaks to the H-4*u* proton doublets at 4.57 (minor, J = 7.6 Hz) and 4.47 (major, J = 7.6 Hz), respectively. Since the region between 4.2 and 4.8 ppm is rather crowded and primarily consists of higher order, distorted proton signals, this is of course reflected in somewhat more poorly resolved COSY-correlations within that area. However, the following off-diagonal peaks could clearly be distinguished: a correlation between H-4*u* min and δ 4.39 (*d*, J = 8.8 Hz), and one from δ 4.39 to δ 4.55 (*m*) and a correlation between H-4*u* and the doublet at δ 4.30 (J = 9.2 Hz), which is again coupled to a multiplet at δ 4.42. Upon careful inspection of an expansion of the regions, δ 4.42/4.47 and δ 4.55/4.57 cross-peaks also became apparent.

HETCOR analysis then revealed the corresponding carbon signals. Protons at δ 4.42, 4.55, 4.30 and 4.39 correlated to δ 73.23 (major), 73.02 (minor), 83.54 (major) and 83.68 (minor) carbons, respectively. Thus, it follows that the δ 73.02 and 73.23 signals are from C-3*u* min and C-3*u*, and those at δ 83.54 and 83.68 from C-2*u* and C-2*u* min, respectively. These assignments will be confirmed by several additional correlations in other spectra and complete the unambiguous assignment of the isolated aliphatic systems.

It is now necessary to use a long-range technique to link it to the remaining aromatic clusters. As the long-range HETCOR also provides the couplings to the quaternary carbons and, thus, supplementary information to the LRCOSY spectra, this approach was chosen to illustrate the spectral interpretation.

First of all, the long-range HETCOR spectrum affords data to check and confirm the previous assignments and allows sequence assignment of the various isolated spin systems, because such spectra only show signals for through-bond correlations over two and three bonds. A correlation is observed between the protons at δ 2.79 (H-4*t*) and 2.89 (H-4*t* min) and the carbons at δ 68.31 and 68.10, respectively, confirming the attribution of C-3*t* and C-3*t* min. In a similar way, the following correlations give additional proof of the assignments cited: H-4*t* (δ 2.79 ppm)/δ 82.07 ($\rightarrow$C-2*t*); H-4*t* min (δ 2.89 ppm)/δ 82.89 ($\rightarrow$C-2*t* min). There

Table 1. NMR assignments and correlations for procyanidin B3

	^{13}C	^{1}H	COSY	LRCOSY	HETCOR	LRHETCOR	HMQC	HMBC
8a*u*	158.47					4.47 5.82		4.47 5.82
8a*u* min	158.36					4.57 5.85 or 5.86		4.57 5.85 or 5.86
7*u* min	157.30					5.85 or 5.86		5.85 or 5.86 8.05
5*u* min	157.10					5.85 or 5.86		6.56
7*u*	157.02					*		5.93 8.08
5*u*	156.89					5.93		6.71
5*t* min	155.45					*		2.62 2.89 6.04 8.20
5*t*	155.41					6.16		2.54 2.79 6.16 8.22
7*t* min	155.29					4.57 6.04		7.59
7*t*	154.90					6.16		4.57
8a*t*	154.69					2.79 4.47		4.47 4.59
8a*t* min	154.59					2.89		4.57 4.74
4′*u* min	145.68					6.99		6.84 6.99
3′*t* min	145.65					6.76		6.76
4′*t* min	145.55					6.88		6.88 7.01
3′*u* min	145.43					6.79		6.79
4′*u*	145.40					6.78		6.51 6.78
4′*t*	145.19					6.28 6.64		6.28 6.64
3′*u*	144.97					6.51 6.679		6.679 6.78
3′*t*	144.85					6.683		6.683
1′*u*	132.44					6.679		6.679
1′*u* min	132.29					6.79		6.79
1′*t* min	131.73					6.76		6.76
1′*t*	131.43					6.683		6.683
6′*u* min	120.69	6.84 *dd* (8.4; 2 Hz)	6.79 6.99	6.99	6.84	6.99	6.84	6.99
6′*u*	120.45	6.51 *dd* (8.4; 2 Hz)	6.679 6.78	4.30 6.679	6.51	6.78	6.51	4.30 6.78
6′*t* min	120.00	6.88 *dd* (8; 2 Hz)	6.76 7.01	6.76	6.88	7.01	6.88	4.74 7.01
6′*t*	119.52	6.28 *dd* (8; 2 Hz)	6.64 6.683	6.683	6.28	6.64	6.28	4.59 6.64
2′*u*	116.07	6.78 *d* (2 Hz)	6.51	4.30 6.679	6.78	*	6.78	4.30 6.51
2′*u* min	115.87	6.99 *d* (2 Hz)	6.84	4.39 6.84	6.99	*	6.99	6.84
5′*t*	115.71	6.683 *d* (8 Hz)	6.28	6.28	6.68	*	6.683	*
5′*t* min	115.67	6.76 *d* (8 Hz)	7.01	6.88 7.01	6.76	*	6.76	*
5′*u*	115.55	6.679 *d* (8 Hz)	6.78	6.51 6.78	6.79	*	6.679	*
5′*u* min	115.51	6.79 *d* (8 Hz)	6.84	—	—	*	6.79	*
2′*t*	115.14	6.64 *d* (2 Hz)	6.28	—	6.64	*	6.64	4.59 6.28
2′*t* min	114.97	7.01 *d* (2 Hz)	6.88	4.74 6.76	7.01	*	7.01	4.47 6.88
8*t*	107.65					4.47 6.16		4.47 6.16
8*t* min	107.54					4.57 6.04		6.04 7.59
4a*u*	106.46					4.47 5.82 5.93		4.47 5.82 5.93 6.71

4a*u* min	106.08					4.57 5.85 or 5.86		5.85 or 5.86 6.56
4a*t*	102.23					2.54 2.79 6.16		6.16 8.22
4a*t* min	100.55					2.62 2.89 6.04		6.04 8.20
6*t* min	97.47	6.04 *s*	—	*	6.04	6.04	6.04	7.59 (8.05)
6*u* min	97.22	5.85 *d* (2.4 Hz)	*	4.57	5.85	5.85 or 5.86	5.85	6.56
6*u*	96.98	5.93 *d* (2.4 Hz)	5.82	4.47 5.82	5.93	*	5.93	6.71
8*u*	96.67	5.82 *d* (2.4 Hz)	5.93	4.47 5.93	5.82	5.93	5.82	5.93
8*u* min	96.12	5.86 *d* (2.4 Hz)	*	4.57	5.86	5.85 or 5.86	5.86	5.85
6*t*	96.09	6.16 *s*	—	*	6.16	6.16	6.16	8.05
2*u* min	83.68	4.39 *d* (8.8 Hz)	4.55	6.99	4.39	6.99	4.39	6.84 6.99
2*u*	83.54	4.30 *d* (9.2 Hz)	4.42	4.47 6.51 6.78	4.30	6.78	4.30	6.51 6.78
2*t* min	82.89	4.74 *d* (7.6 Hz)	4.08	7.01	4.74	2.62 2.89 7.01	4.74	2.62 2.89 6.88 7.01
2*t*	82.07	4.59 *d* (7.2 Hz)	3.83	*	4.59	2.54 2.79 6.64	4.59	2.54 2.79 6.28 6.64
3*u*	73.23	4.42 *m*	4.30 4.47	*	4.42	*	4.42	4.47
3*u* min	73.02	4.55 *m*	4.39 4.57	*	4.55	*	4.55	4.57
3*t*	68.31	3.83 *m*	2.54 2.79 4.59	*	3.83	2.54 2.79	3.83	2.54 2.79 4.59
3*t* min	68.10	4.08 *m*	2.62 2.89 4.74	*	4.08	2.62 2.89	4.08	2.62 2.89 4.74
4*u*	38.09	4.47 *d* (7.6 Hz)	4.42	4.30 5.82 5.93	4.47	4.42	4.47	4.42
4*u* min	37.97	4.57 *d* (7.6 Hz)	4.55	5.85 or 5.86	4.57	4.55	4.57	*
4*t*	28.82	α-H 2.79 *dd* (16.4, 5.6 Hz)	2.54 3.83	*	*	*	2.54 2.79	4.59 or 4.74
		β-H 2.54 *dd* (16.4; 8 Hz)	2.79 3.83	*	*	*		
4*t* min	28.64	α-H 2.89 *dd* (16; 5.6 Hz)	2.62 4.08	*	*	*	2.62 2.89	4.74 or 4.59
		β-H 2.62 *dd* (16; 8 Hz)	2.89 4.08	*	*	*		
70H *t* min		7.59 *s*						97.47 107.54
70H *u* min		8.05 *s*						157.30
70H *u*		8.08 *s*						157.02
70H *t*		8.56 *s*						107.65
50H *u* min		6.56 *br s*						97.22 106.08 157.10
50H *u*		6.71 *br s*						96.98 106.46 156.89
50H *t* min		8.20 *s*						97.47 100.55 155.45
50H *t*		8.22 *s*						96.09 102.23 155.41

*Correlations not clear.

was also a non-resolved cross-peak of δ 73.02 and 73.23 to H-4*u* (δ 4.47) and *H*-4*u* min (δ4.57), indicating C-3*u* and *C*-3*u* min carbons, yet not permitting any discrimination between them.

Used as a linkage took of isolated spin systems, the following clues could be observed. The long-range HETCOR showed correlations between H-4*t* and δ 102.23, H 4*t* min and δ 100.55, H-4*u* and both δ 106.46 and 107.65, and H-4*u* min and both δ 106.08 and 107.54. As these are quaternary aromatic carbons not deshielded by any directly coupled phenolic groups, they must belong to the C-4a carbons, together with the 6- or 8-carbon of the same moiety involved in the interflavan linkage; δ 102.23 can then readily be identified as C-4*at* and δ 100.55 as C-4*at* min. Other correlations to δ 106.08 (C-4a*u* min), 106.46 (C-4a*u*), 107.65 (C-8*t*) and 107.54 (C-8*t* min) will provide definitive assignments. As C-4a*t* min correlates with a singlet at δ 6.04 ppm and since the only isolated spin is the free 6- or 8-proton in the terminal unit, it is tentatively attributed to H-6/H-8*t* min at this stage. Identically, the δ 6.16 singlet can be identified as H6/H8*t*. In their turn, these proton singlets (δ 6.04 and 6.16) exhibit cross-peaks with carbons at δ 107.54 and 107.65, respectively. Therefore, these have to be C8/C-6*t* min and C-8/C-6*t*, and δ 106.08 has to be C-4a*u* min and δ 106.46 is C-4a*u*. C-4a*u* min is responsible for other correlations to two proton doublets at δ 5.85 ($J = 2.4$ Hz) and 5.86 ($J = 2.4$ Hz) belonging to the 6- and 8-protons in the upper unit of the minor rotamer. C-4a*u* showed long-range correlations to the protons resonating at δ 5.82 (*d*, $J = 2.4$ Hz) and δ 5.93 (*d*, $J = 2.4$ Hz).

For ease of notation, we will now assume that the two units are indeed 4→8 coupled as a working hypothesis. This assumption will be proven subsequently. Therefore, the following assignments can be made: δ 6.04 H-6*t* min, δ 6.16 H-6*t*, δ 107.54 C-8*t* min and δ 107.65 C-8*t*. From the one-bond HETCOR, it then follows that δ 97.47 is C-6*t* min and δ 96.09 is C-6*t*.

The nature of the ^{1}H three-spin catechol B-ring systems followed from the magnitude of the coupling constants in the 1D spectrum (Table 1), while their assignments and points of attachment were afforded by the COSY and long-range COSY spectra. Hence, in the latter spectrum, the H-2*u* signal displayed two long-range correlations with a double doublet at δ 6.51 (H-6′*u*, $J = 8$ and 2 Hz) and a doublet at δ 6.78 (H-2′*u*, $J = 2$ Hz). H-6′*u* showed a correlation with a doublet, either at δ 6.679 or 6.683 (not unambiguous in the long-range COSY) (H-5′*u*, $J = 8$ Hz). Similar correlations of H-2*u* min, H-2*t* and H-2*t* min allowed unambiguous assignment of H-2′, H-6′ and H-5′ of the corresponding catechol B-ring systems. These assignments were confirmed from the long-range HETCOR, where cross-peaks were observed between the 2′ and 6′ carbons and H-2′.

The remaining four C–H signals from δ 96.12 to 97.22 are due to the 6- and 8-carbons of the upper unit. HETCOR reveals correlations of δ 96.12 with a doublet at δ 5.86 ($J = 2.4$ Hz), of δ 96.67 with δ 5.82 (*d*, $J = 2.4$ Hz), of δ 96.98 with δ 5.93 (*d*, $J = 2.4$ Hz) and of δ 97.22 with δ 5.85 (*d*, $J = 2.4$ Hz). Long-range COSY shows cross-peaks of H-4*u*, with both δ 5.82 and 5.93, which also have a mutual cross-peak. Therefore, we can decide that δ 5.82 and 5.93 are of the 6- and 8-protons of the major rotamer and δ 5.85 and 5.86 stem from their counterparts in the minor rotamer. However, distinction between these two (6 or 8) was not yet possible at this point.

Thus, only quaternary aromatic carbons remain to be identified from the long-range HETCOR spectrum. The higher field four-peak cluster around 132 ppm shows ^{1}H–^{13}C long-range correlations to the H-5′ protons and belong to the non-oxygen bearing 1′-carbons. The signal at δ 132.44 is correlated with H-5′*u* and is, thus, C-1′*u*. H-5′*u* min has a cross-peak with δ 132.29 (→C1′*u* min), 5′*t* min with δ 131.73 (→C-1′*t* min) and 5′*t* with δ 131.43 (C-1′*t*).

Next, there is a very crowded area around 145 ppm containing eight signals. Cross-peaks are found to 2′, 5′ and 6′ protons, indicating that these belong to the 3′ and 4′-carbons. The long-range HETCOR, optimized for a relatively large J_n CH of 10 Hz, revealed the following correlations, from which the conclusions stated were drawn. However, because of the very small relative chemical shift differences, assignments were only tentative at this stage. Later on, they were confirmed by the results of the HMBC-experiment. H-2′*u* min correlated with δ 145.68 (→*C*-4′*u* min), H-5′*t* min with δ 145.65 (→3′*t* min), H-6′*t* min with δ 145.55 (→C-4′*t* min) and H-5′*u* min with δ 145.43 (→*C*-3′*u* min). H-2′*u* showed cross-peaks with δ 145.40 (→C-4′*u*), H-6′*t* and H-2′*t* with δ 145.19 (→C-4′*t*), H-6′*u* and H-5′*u* with δ 144.97 (→C-3′*u*) and *H*-5′*t* with δ 144.85 (→C-3′*t*).

The last cluster, situated between δ 154.59 and 158.47 ppm contains 12 signals, belonging to the 5, 7 and 8a carbons. The carbon at δ 154.59 shows a correlation to both H-4*u* min and H-4*t* min and is, therefore, concluded to be C-8a*t* min. Identically, the carbon at δ 154.69, correlating to H-4*u* and H-4*t* is assigned as *C*-8a*t*. The carbon signal at δ 154.90 shows a cross-peak with H-6*t* and H-4*u*. It should thus be attributed to C-7*t*. Again, in the minor rotamer, there is a similar situation; the signal at δ 155.29 has correlations to H-4*u* min and H-6*t* min and is, thus, C-7*t* min. The following two signals at δ 155.41 and 155.45 could not be distinguished from one another; they showed correlations to H-6*t*, H-4*t* and H-4*t* min. Hence, these signals have to belong to the carbons C-5*t* of both rotamers. The remaining six signals all have correlations with the 6 or 8 signals of the upper unit. Their assignments are discussed below.

Addition of a trace of $Cd(NO_3)_2$ to a 50 mg procyanidin B3 solution in acetone-d_6, caused the broad OH-absorption peak to separate into sharp, distinct signals in the low-field region of the spectrum between 7.5 and 8.6 ppm. This sample was then used for indirect detection experiments at 600 MHz. The

long-range C–H correlation technique, HMBC, then afforded the diagnostic cross-peaks needed for 6/8 discrimination.

The one-bond HMQC experiment confirmed the results displayed by the HETCOR spectrum and attributions previously obtained from the long-range HETCOR, proved correct from HMBC correlations (cf. Table 1). Only the correlations leading to 6/8 discrimination and assignments for the quaternary A and D-ring carbons will be discussed here.

The OH-proton at δ 8.22 displayed cross-peaks with the firmly established C-6*t* and C-4a*t* and is, thus, 5-OH*t*. Similarly, that at δ 8.20, correlating with C-6*t* min and C-4a*t* min is 5-OH*t* min. The spectrum also shows correlations between the signal at δ 7.59 and the signals at δ 97.47 (C-6*t* min) and δ 107.54 (C-8*t* min), permitting assignment of the signal to 7-OH*t* min. Its major rotamer analogue (7-OH*t*) was found at δ 8.56, correlating with C-8*t*. Furthermore, the peak at δ 6.56 is correlated to C-4a*u* min and to δ 97.22, being either C-6*u* min or C-8*u* min. The clearly shown connectivity of the OH-signal at δ 6.56 to C-4a*u* min permitted assignment of this absorption to 5-OH*u* min and, hence, of the δ 97.22 resonance to C-6*u* min. Identically, δ 6.71 was assigned as 5-OH*u* owing to correlations to C-4a*u* and C-6*u* (δ 96.98). Subsequently, correlations in both the HETCOR and HMQC experiments then identified H-6*u* at δ 5.93 and H-6*u* min at δ 5.85. Long-range correlations in the HMBC spectrum from the signal at δ 96.67 to H-6*u*, and from δ 96.12 to H-6*u* min, attributed them to C-8*u* and C-8*u* min, respectively. With these carbon resonances assigned, the one-bond techniques then permitted assignment of proton signals at δ 5.86 and 5.82 to H-8*u* min and H-8*u*, respectively.

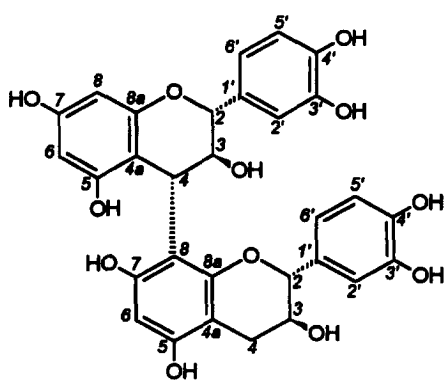
Subsequently, assignment of the remaining quaternary A and D-ring carbons became possible by analysis of the long-range HETCOR or HMBC spectra. The signals at δ 155.41 and 155.45 could be distinguished, because the former correlated with H-6*t*, the H-4*t*s and with 5-OH*t* and is, thus, C-5*t*. Similar correlations identified the latter as C-5*t* min. Another experiment showed correlation between H-4*u* and H-8*u* and the signal at δ 158.47, indicating this to be C-8a*u*. Analogously, assignment of the carbon resonance at δ 158.36 to C-8a*u* min was facilitated by connectivities with H-4*u* min and H-8*u* min or H-6*u* min. The signal at δ 156.89 is correlated with 5-OH*u* and the one at δ 157.10 ppm with 5-OH*u* min; they were assigned to C-5*u* and C-5*u* min, respectively. Since the resonance at δ 157.02 shows only one correlation to H-6*u* in the HMBC spectrum, this signal was assigned to C-7*u*; it is also correlated to the OH-proton at 8.08 ppm (→7-OH*u*). The signal at δ 157.30 correlating with one of the minor 6/8 H-signals corresponds then to C-7*u* min; the additional cross-peak can be assigned to δ 8.05 (→7-OH*u* min).

The carbon and proton spectra of procyanidin B3 are thus completely assigned. Confirmatory proof of the (4→8) interflavanoid bond was readily demonstrated from the simultaneous HMBC-correlations from C-8a*t* to H-2*t* and H-4*u* [13]. This was observed for both rotameric forms. The power of this experiment lies in its ability to discriminate between signals due to chain extender and lower terminal units in both rotameric forms for each interflavan bond. The above example serves to illustrate the considerable power of C–H heteronuclear correlation experiments in the study of free phenolic proanthocyanidins.

EXPERIMENTAL

Biomimetic synthesis of procyanidin B3. (+)-Taxifolin was isolated from *Pseudotsuga menziesii* (Mirb.) Franco. (+)-Catechin was obtained from Sigma. (+)-Taxifolin (200 mg) was dissolved in a minimal vol. of EtOH and treated with an excess of $NaBH_4$ (200 mg) dissolved in EtOH added dropwise in *ca* 30 min under N_2 with continuous stirring. Subsequently, excess (+)-catechin (600 mg) dissolved in EtOH was added to the reaction mixt. The pH was adjusted to 2 with aq. HOAc (2M). After 1 hr reaction time, a sample was analysed by TLC and, subsequently, at 30 min intervals, for the presence of trimers. When the first traces of trimers appeared, the reaction was stopped and the procyanidins immediately extracted with EtOAc. The EtOAc layer was evapd to dryness under red. pres. and subjected to CC on Sephadex LH-20 using a *n*-PrOH–MeOH gradient varying from 75% *n*-PrOH to 100% MeOH. The second of five pooled frs afforded after two more chromatographic steps on Sephadex LH-20 with the same gradient, a slightly brown-coloured amorphous powder (98 mg), producing a $[MH]^-$ ion at *m/z* 577 in negative ion FAB-MS. The product was identified as procyanidin B3 or catechin-(4α→8)-catechin

NMR experiments. NMR were recorded on a Varian Unity 400 instrument (^{1}H: 399.9 MHz ^{13}C: 100.6 MHz) and a Bruker AM-600 spectrometer (^{1}H: 600.1 MHz ^{13}C: 150.9 HMz), using 5-mm tubes, and locked to the deuterium resonance of the solvent, acetone-d_6. Chemical shifts are reported in ppm on the δ scale; spectra are referenced to the solvent. The temp. was maintained at 303 K and 300 K, respectively. Standard software packages were used to record 1D ^{1}H and ^{13}C (broad-band



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decoupled, APT and DEPT-135) spectra. 2D ^{13}C-detected HETCOR (one-bond correlation) and long-range HETCOR spectra were recorded on the Varian spectrometer using standard software, while 2D ^{1}H-detected spectra (HMQC and HMBC) were implemented and recorded on the Bruker instrument.

Acknowledgements—One of the authors (T. De B.) is a Research Assistant of the National Fund for Scientific Research (Belgium). This work was financially supported by the Flemish Government (Concerted Action No. 92/94-09).

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