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Structure and Conformation of the Procyanidin B-2 Dimer

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The structure of the proanthocyanidin B-2 dimer was proved to be (−)epicatechin-(4β-8)-(−)epicatechin by two-dimensional heteronuclear NMR spectroscopy. The compound adopts two conformations in solution, in which the interflavan dihedral angles are roughly ±90°. The preferred conformation of the pyran ring is a half-chair with the 3′,4′-dihydroxyphenyl substituents in pseudo-equatorial positions. © 1997 John Wiley & Sons, Ltd.

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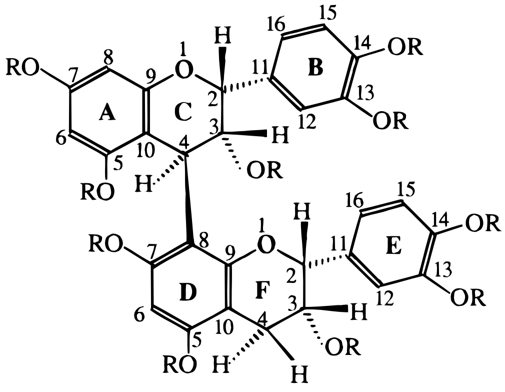
INTRODUCTION

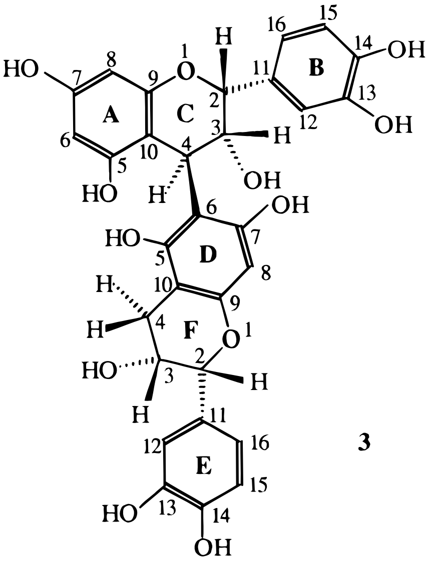
B-type[1] proanthocyanidins are widely distributed in nature, and have been attributed a range of therapeutic effects, often being considered as the active ingredients of herbal medicines.[2] Procyanidin B-2 (**1**) is extracted from hawthorn, and is a dimer of (−)epicatechin. It has been extensively studied[1,3–9] but there has as yet been no conclusive proof whether the flavan junction is (4–8) (as in **1**) or (4–6) (as in **3**), or of its conformation in solution. The covalent structure of proanthocyanidins has previously been elucidated using chemical shifts,[3,4,7,10,11] from partial thiolytic degradation[11] or using arguments based on chemical reactivity.[1] All these approaches have been called into question. Thus, exceptions to the general chemical shift rule have been noted,[12] and the demonstration of side-reactions during thiolysis has reduced the utility of the model.[13] Therefore, more recently heteronuclear NMR methods have been the preferred methods for the structure elucidation of anthocyanidins.[14–18] We have therefore undertaken a heteronuclear NMR study of B-2, with the aim of reliably establishing the covalent structure. This study also provided parameters that allow us to deduce the solution conformation of B-2.

RESULTS AND DISCUSSION

The ^{1}H and ^{13}C NMR spectra of B-2 are broad at room temperature in all available solvents. The appearance of the spectra varies with temperature, solvent and field strength, suggesting the presence of conformational



1 R=H
2 R=Ac



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exchange. The most useful spectra of underivatized B-2 (**1**) were obtained at 500 MHz in $(CD_3)_2CO$ at low temperature (268 K), under which conditions the conformational exchange is almost frozen out, as indicated by reasonably sharp signals for the major component. However, only one set of signals could be assigned, implying that the signals for the minor component(s) are still broadened. Measurements at 226 K showed that there is only one minor component present, with a concentration about 6% of that of the major component, which is consistent with the broadness of its signals at 268 K. The decaacetate derivative of B-2 (**2**) has a slower conformational exchange, with two clearly identifiable conformers in a ratio of 1 : 3, and gives good quality spectra even at room temperature: the assignment of **2** was therefore carried out at room temperature. We report below the assignments at 295 K.

Assignments and structure determination

NMR assignments were made using a combination of homonuclear correlation (COSY) experiments and heteronuclear one-bond (HSQC/HMQC) and multiple bond (HMBC) correlations. The starting points for the assignment were the H-4 protons, which appear at the unique and readily identifiable chemical shift of around 2.8 ppm, and the C-4 carbons, which resonate at around 36 ppm for ring C and 28 ppm for ring F. (The nomenclature used in the rest of this paper is X-*n*Y, where X is the atom type, *n* is the numbering of the atom given in structures **1** and **2**, and Y is the ring designation; thus, the two carbons in position 4 are C-4C and C-4F.) The assignment process was simplified by presentation of the HMBC spectra as stacked plots through selected proton resonances (Fig. 1). The assignments are presented in Tables 1 and 2. The key question of whether the flavan junction is (4–8) or (4-6) was answered by considering the long-range correlations to C-9 of ring D, which are to H-2 and H-4 of ring F (thereby confirming the carbon resonance as C-9 rather than C-5) and to H-4 of ring C, which is only possible if the junction is (4–8). A similar strategy has been used in other studies of procyanidins.[14]

The (4–8) ring junction was further confirmed by the spectra from the decaacetate derivative. Acetylation is expected to produce an upfield shift of about 8 ppm on any aromatic carbon bearing a hydroxyl group. In the underivatized **1**, H-4C showed correlations to carbons at 72.6, 76.5, 99.5, 106.7, 153.8, 155.6, 157.4 and 158.0 ppm. In the derivatized **2**, the correlations were to carbons at 71.0, 73.5, 111.0, 116.2, 147.2, 148.6 153.9 and 154.8 ppm. This implies that only two of the phenolic carbons with long-range coupling to H-4C have become acetylated [consistent with the (4–8) coupling], rather than three, as expected for a (4–6) coupling.

There has been considerable discussion in the literature over the assignment of H-6/H-8 and C-6/C-8 in catechins and epicatechins (for examples, see Refs 17 and 18 and literature cited therein). The positions of these signals are solvent dependent, and also change significantly on derivatization of the procyanidins, but it has recently been concluded that in general H-8 and C-8 resonate to higher field than H-6 and C-6, respec-

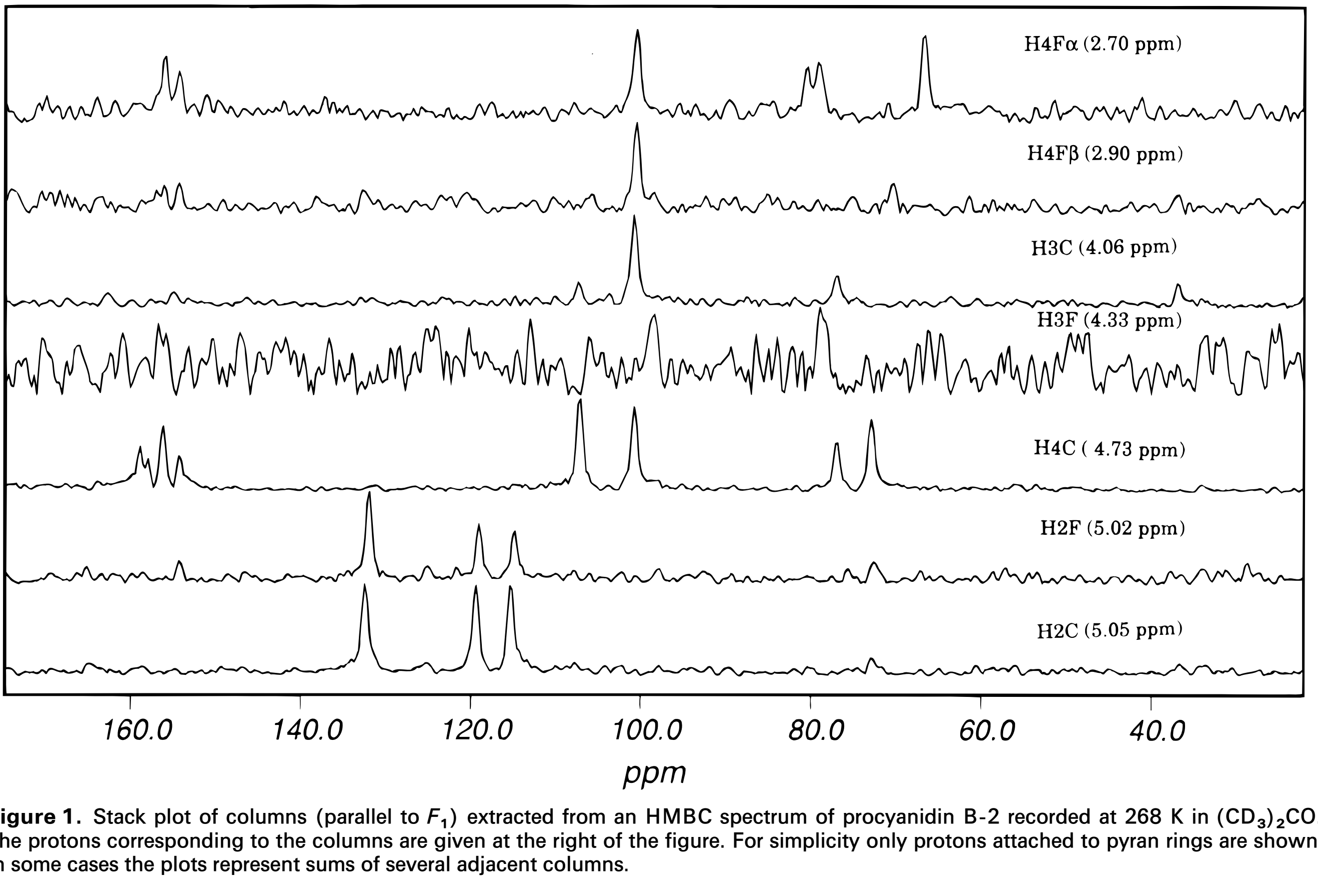

Figure 1. Stack plot of columns (parallel to F_1) extracted from an HMBC spectrum of procyanidin B-2 recorded at 268 K in $(CD_3)_2CO$. The protons corresponding to the columns are given at the right of the figure. For simplicity only protons attached to pyran rings are shown. In some cases the plots represent sums of several adjacent columns.

Table 1. ^{1}H chemical shift assignments for 1 and 2 at 295 K[a]

Ring	Proton	**1**, 500 MHz, $(CD_3)_2CO$	**2**, 400 MHz, $CDCl_3$ Major conformer	**2**, 400 MHz, $CDCl_3$ Minor conformer
C	H-2C	5.11 (bs)	5.56 (bs)	5.39 (bs)
	H-3C	4.02 (bs)	5.15 (t, 1.8)	5.31 (t, 1.5)
	H-4C	4.74 (bs)	4.45 (d, 2.0)	4.63 (bs)
F	H-2F	4.97 (bs)	4.53 (bd)	5.24 (bs)
	H-3F	4.31 (bs)	5.09 (m)	5.53 (m)
	H-4Fα	2.74 (dd, 3.8, 16.6)	2.84 (dd 1.6, 18.4)	3.01 (dd, 2.2, 18.0)
	H-4Fβ	2.91 (dd, 4.7, 16.7)	2.92 (dd, 4.8, 18.4)	3.05 (dd, 4.4, 18.0)
A	H-6A	6.01 (bs)	6.22 (d, 2.3)	6.63 (d, 2.3)
	H-8A	6.00 (bs)	5.95 (d, 2.3)	6.75 (d, 2.3)
D	H-6D	5.97 (bs)	6.64 (s)	6.59 (s)
B	H-12B	6.99 (m)	7.34 (d, 2.0)	
	H-15B	6.73 (m)	7.16 (d, 8.4)	
	H-16B	6.99 (m)	7.24 (m)	
E	H-12E	6.99 (m)	7.00 (d, 2.0)	
	H-15E	6.71 (m)	7.02 (d, 8.2)	
	H-16E	6.99 (m)	6.87 (dd, 8.3, 2.0)	
	CH_3CO		1.86–2.35	1.54–2.35

[a] bs = Broad singlet, bd = broad doublet, d = doublet, t = triplet, m = multiplet. For resolved multiplets, couplings are given in Hz. Shifts are given relative to the internal $(CD_3)_2CO$ residual proton signal at 2.04 ppm or $CDCl_3$ at 7.25 ppm.

Table 2. ^{13}C chemical shift assignments for 1 and 2 at 295 K[a]

Ring	Carbon	**1**, 125 MHz, $(CD_3)_2CO$	**2**, 100 MHz, $CDCl_3$ Major conformer	**2**, 100 MHz, $CDCl_3$ Minor conformer
C	C-2C	76.6	73.5	74.3
	C-3C	72.6	71.0	70.5
	C-4C	36.5	34.0	34.1
F	C-2F	79.0	77.2	76.6
	C-3F	66.1	66.9	66.3
	C-4F	28.6	26.7	26.5
A	C-5A	158.0	147.2	149.1
	C-6A	96.3	108.6	108.9
	C-7A	157.2	148.6	149.1
	C-8A	95.9	107.2	108.1
	C-9A	155.6	154.8	154.4
	C-10A	100.3	111.0	111.2
D	C-5D	155.5	147.2	146.7
	C-6D	96.5	110.2	110.8
	C-7D	157.4	148.6	146.7
	C-8D	106.7	116.2	117.2
	C-9D	153.8	153.9	151.3
	C-10D	100.3	111.3	109.3
B	C-11B	132.0	136.1	
	C-12B	115.1	122.1	
	C-13B	145.1	140.9	
	C-14B	145.1	141.4	
	C-15B	115.3	123.0	
	C-16B	119.0	124.4	
E	C-11E	130.4	133.9	
	C-12E	115.8	122.4	
	C-13E	145.1	141.1	
	C-14E	145.1	141.1	
	C-15E	115.1	122.7	
	C-16E	120.3	125.0	
	Acetate CH_3		19.8–21.2	
	Acetate CO		167.9–170.4	

[a] Shifts are given relative to the internal $(CD_3)_2CO$ signal at 29.8 ppm or the $CDCl_8$ signal at 77.0 ppm.

tively, in the underivatized compounds, but resonate to lower field after methylation or acetylation.[17] Procyanidin B-2 has somewhat atypical chemical shifts, in that the resonances for H-6 and H-8 are very close together. We find that in the underivatized compound **1**, H-8A and C-8A do indeed resonate to higher field of H-6A and C-6A. However, in the acetylated compound **2**, the relative shifts are conformation dependent: in the major component H-8A and C-8A resonate to high field of H-6A and C-6A, as they do in the underivatized compound, but in the minor component H-8A resonates to low field of H-6A. Hence it is unwise to base assignments or structural conclusions on chemical shift arguments.

Conformational analysis

The NMR spectra of B-2 provide ample evidence of conformational exchange. From consideration of the structure, two different types of conformational exchange are possible: restricted rotation about the C-4—C-8 interflavan bond, or slow ring interconversion of either or both of the pyran rings.[19–21] Several lines of evidence favour the former possibility, in particular, the observation that the $^3J_{2,3}$ and $^3J_{3,4}$ couplings are very similar and small in both conformers of **2** and the observation that conformational exchange is markedly slower in the peracetate derivative, which would be expected in the former case but not particularly expected in the latter.

In order to clarify further details of the exchange process, molecular mechanics calculations were carried out using the MM2 force field. Two local minima were found which differ in their interflavan dihedral angles by roughly 180°, as listed in Table 3. The result is in line with previous calculations.[8,9] The resultant conformations are shown in Fig. 2, and their calculated and

Table 3. Local minima calculated for procyanide B-2

Compound	Interflavan angle (°)[a]
1	98.2
1	−80.8
2	95.2
2	−83.5

[a] The angle is defined as ϕ = C-3C—C-4C—C-8D—C-9D.

observed coupling constants are compared in Table 4. All conformations have the pyran rings in a half-chair geometry, with the 3′,4′-dihydroxyphenyl rings B and E in quasi-equatorial positions (Fig. 3). This gives very small calculated coupling constants $^3J_{2,3}$ and $^3J_{3,4}$ in ring C, as observed experimentally. Other geometries give rise to much larger coupling constants; for example, a half-chair conformation of ring C with ring B in a quasi-axial position has a calculated $^3J_{2,3}$ of about 4.5 Hz. We therefore conclude that the B-2 dimer has a conformation predominantly as shown in Fig. 2, with the dihydroxyphenyl rings almost entirely in quasi-equatorial positions. This result is in accord with Hemingway *et al.*,[17] who concluded that (−)-epicatechins with bulky substituents in the 4β-position (as in B-2 and a large number of naturally occurring procyanidin oligomers) have the dihydroxyphenyl rings predominantly quasi-equatorial in ring C, despite this putting the 4β-substituent in a quasi-axial position.

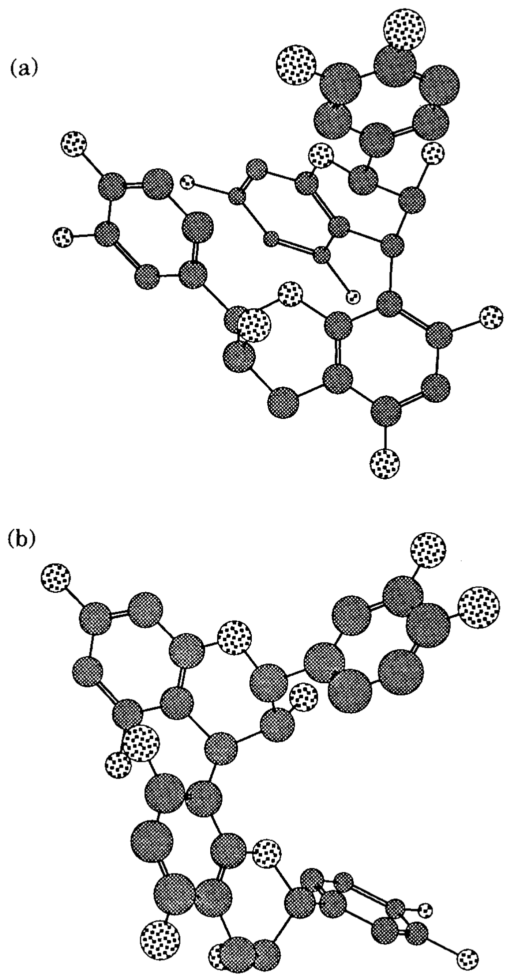

Figure 2. Energy-minimized structures calculated for procyanidin B-2. Shaded atoms are carbon and dotted atoms are oxygen. The radii of atoms are depth coded. ϕ = (a) 98.2° and (b) 80.8°.

EXPERIMENTAL

Procyanidin B-2 was purified from hawthorn berries (*Crataegus monogyma*) using literature methods.[3] It was acetylated using acetic anhydride in pyridine, followed by purification on a silica gel column.[1] NMR spectra were acquired using Bruker AMX500 (**1**) and AMX400 (**2**) spectrometers with standard Bruker software, and processed using FELIX (Biosym Technologies) on a Silicon Graphics workstation. Two-dimensional experiments were typically recorded into 1000 4K complex files using time-proportional phase incrementation with spectral widths of 12.5 kHz (F_2, ^{1}H) and 17.5 kHz (F_1, ^{13}C), and processed using sine-bell window functions.

Table 4. Comparison of observed and calculated coupling constants (Hz) for procyanidin B-2[a]

		1				**2**				
			Calc.				Calc.		Obs.	
Ring	Coupling	$\phi > 0$	$\phi < 0$	Av.[b]	Obs.	$\phi > 0$	$\phi < 0$	Av.[b]	Maj.	Min.
C	$^3J_{2,3}$	0.4	0.4	0.4	[c]	0.6	0.7	0.7	[c]	[c]
	$^3J_{3,4}$	1.3	1.1	1.2	[c]	0.9	0.7	0.8	1.8	1.5
F	$^3J_{2,3}$	0.4	0.4	0.4	[c]	0.6	0.8	0.7	[c]	[c]
	$^3J_{3,4\alpha}$	2.8	2.7	2.8	3.8[d]	3.1	2.2	2.7	1.6	2.2
	$^3J_{3,4\beta}$	3.2	3.3	3.3	4.7[d]	2.9	4.1	3.5	4.8	4.4

[a] For the definition of ϕ, see Table 3.
[b] The average is a simple mean of the two preceding values, for illustrative purposes.
[c] Too small to measure.
[d] These values are likely to be imprecise, because of the large linewidths in **1** which make measurement of splittings difficult.

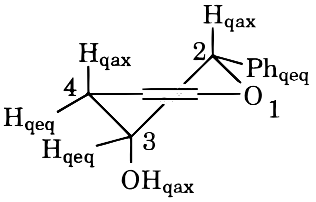

Figure 3. The preferred conformation of the pyran rings. The figure illustrates ring F; in ring C the H-4$_{qax}$ proton is substituted.

Initial models of **1** and **2** were built using MacroModel 4.5 pre-minimized using the Polak–Ribiere conjugate gradient method, with the MM2 force field (1987 parameters)[22], and subjected to a grid search with a step size of 10° to find local minima. The structures were then minimized using the truncated Newton conjugate gradient method, followed by a full matrix Newton Raphson method. Coupling constants were calculated using Altona's equation[23] within MacroModel.

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