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PMR SPECTRA OF NATURAL ACYLATED ANTHOCYANINS

DETERMINATION OF STEREOSTRUCTURE OF AWOBANIN, SHISONIN AND VIOLANIN

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Structure of natural acylated anthocyanins are usually determined by chemical degradation into their components such as anthocyanidin, sugar and a cinnamic acid derivative.[1] The structures proposed from these experiments have more or less ambiguities on the anomeric configuration of the sugar and on the position of attachment and geometrical configuration of the double bond of the acyl moiety. In addition, molecular composition of such anthocyanins is usually very difficult to be determined by ordinary methods including mass spectrometry. Thus, additional small components, if any, are liable to be overlooked.

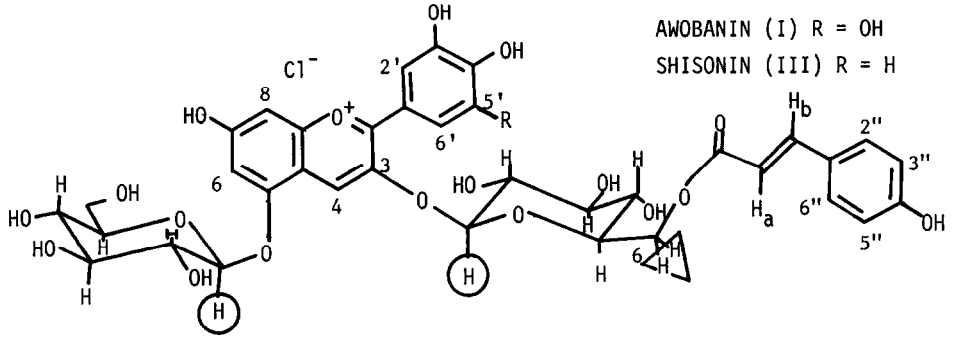
Structure analysis by PMR spectroscopy at a first glance seems to be most promissing, but very little use of PMR techniques has been reported for natural anthocyanins. One of the main reasons would be that by some unknown causes natural anthocyanins give too poor PMR spectra to be used for structural analysis. Nilsson[2] reported PMR spectra of some synthetic anthocyanidins (aglycons of anthocyanins) in CF_3COOD or acidified CD_3SOCD_3. Even in this case, he mentioned that the samples for PMR measurements should be freshly synthesized; re-crystallization makes sample rather impure.

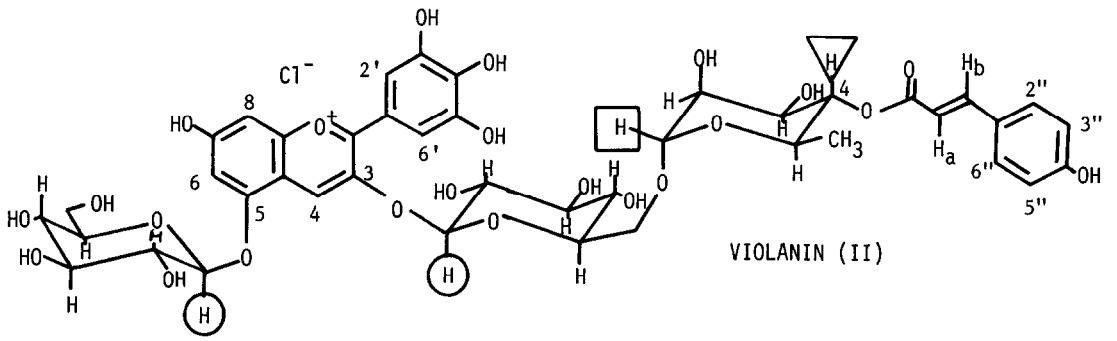
After some trials we have succeeded in obtaining very fine PMR spectra of natural acylated anthocyanins such as awobanin, shisonin and violanin by raising temperature of the probe. By analysis of the spectra we could determine unambiguously complete stereostructure of these pigments. This method would be widely applicable to the structure determination of other natural anthocyanins.

Awobanin is the anthocyanin component of commelinin, the sky-blue color of Commelina communis L. and its structure has been suggested to be delphinidin 3-(p-coumaroyl-D-glucoside)-5-D-glucoside[3,4] without determining the position of attachment of p-coumaroyl group on the glucose, geometry of the double bond, and anomeric configurations of both of the glucosides. We have tried to make clear the former two points by usual hydrogen peroxide oxidation;[5] awobanin gave mainly 6-(trans-p-coumaroyl)glucose, but its cis isomer was also produced, indicating the possibility of cis-trans isomerization during the reaction. Acyl migration[6] from 4 to 6 position is also a possibility. Thus, structural investigation must be carried out using intact anthocyanins.

Pure awobanin chloride was obtained from commelinin by mild acid treatment and chromatography on an Avicel column using AcOH:HCl:H_2O (5:1:40) as eluant. The combined anthocyanin fractions were evaporated in vacuo below 40° and then quickly dried over KOH. Awobanin chloride (5 mg) thus obtained was dissolved in 0.1 ml CD_3OD and the solution dried up in vacuo.

After repetition of this procedure twice, the residue was dissolved in a mixture of CD_3OD (0.3 ml) and DCl in D_2O (20%, 2 μl) and used for PMR measurements (Fig. 1, JEOL JNM-FX 100 FT-NMR Spectrometer, 100 MHz). Although the spectrum at room temp was quite broad, elevation of probe temperature resulted in the beautiful spectrum (upper). Assignment of the signals is shown in it. Spectral analysis: [1] no signals corresponding cis-p-coumaroyl group were observed, [2] that the p-coumaroyl group is attached to the 6 position of glucose was established by a down-field shift about 1 ppm of the two-proton signal at δ 4.44 corresponding to H-6 of the glucose moiety, [3] two doublet signals appeared at δ 5.45 and 5.14 are attributable to the anomeric protons of two molecules of glucose; the coupling constants of both of the signals are 8 Hz, which clearly indicate the β-configuration of these anomeric positions, and [4] signals of aromatic protons support the delphinidin nucleus. Thus, the complete stereostructure of awobanin can be represented as delphinidin 3-(6-O-trans-p-coumaroyl-β-D-glucoside)-5-(β-D-glucoside) (I).





Similar PMR analysis (vide infra) of violanin chloride obtained from Viola tricolor, and shisonin chloride obtained from Perilla ocimoides determined their complete structure to be (II) and (III), respectively. Incidentally, in the literatures structure of violanin[7,8] and shisonin[4,9] had been assigned to delphinidin 3-[6-O-α-L-(p-coumaroyl)rhamnosyl-D-glucoside]-5-D-glucoside[11] and cyanidin 3-(6-O-p-coumaroyl-D-glucoside)-5-D-glucoside, respectively.

PMR spec. of violanin chloride (II) (13 mg in 0.3 ml CD_3OD containing 2 μl of 20% DCl in D_2O, at 70°) δ 8.86 (1H,s,H-4), 7.71 (2H,s,H-2'and 6'), 7.50 (1H,d,J=16Hz,H_b), 7.34 (2H,$\underline{A_2'B_2'}$, J_{ortho}=8Hz,H-2" and 6"), 6.98 (2H,s,H-6 and 8), 6.76 (2H,$A_2'\underline{B_2'}$,J_{ortho}=8Hz,H-3" and 5"), 6.17

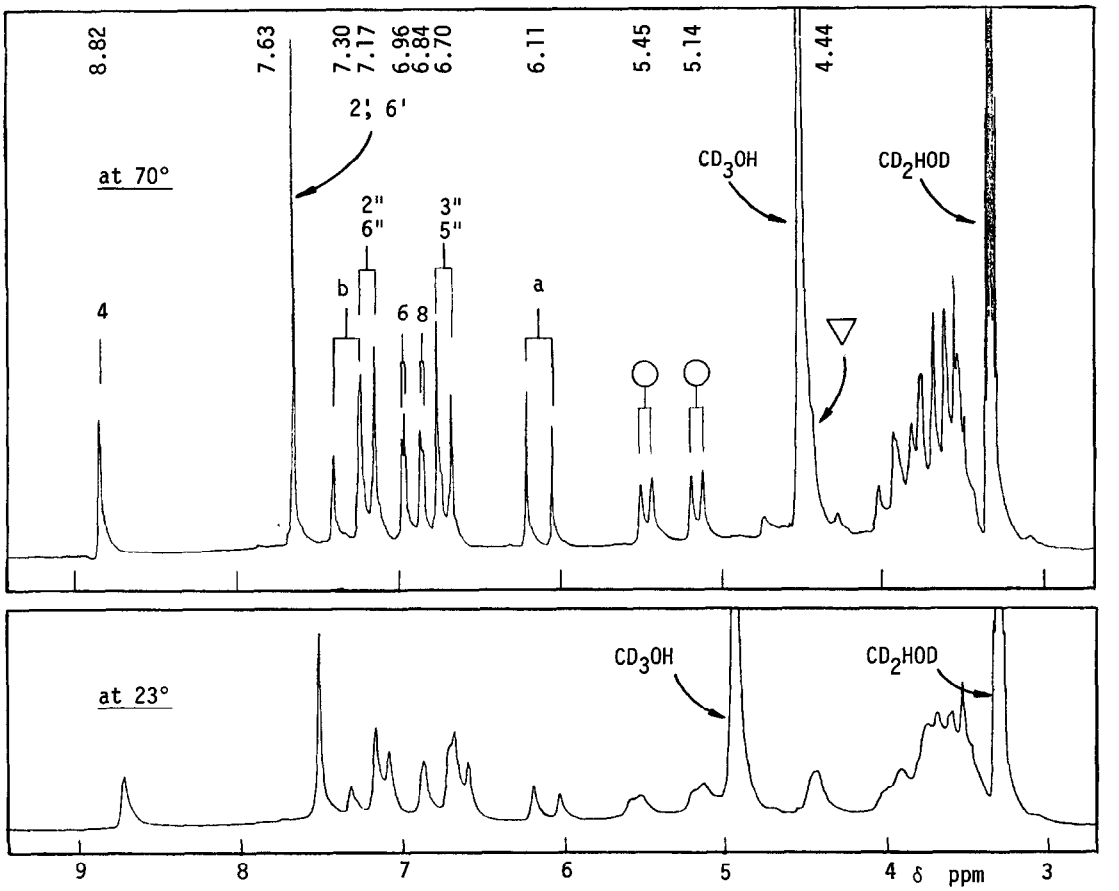


Fig. 1. PMR Spectra of Awobanin Chloride (I) in CD_3OD (64 scans)

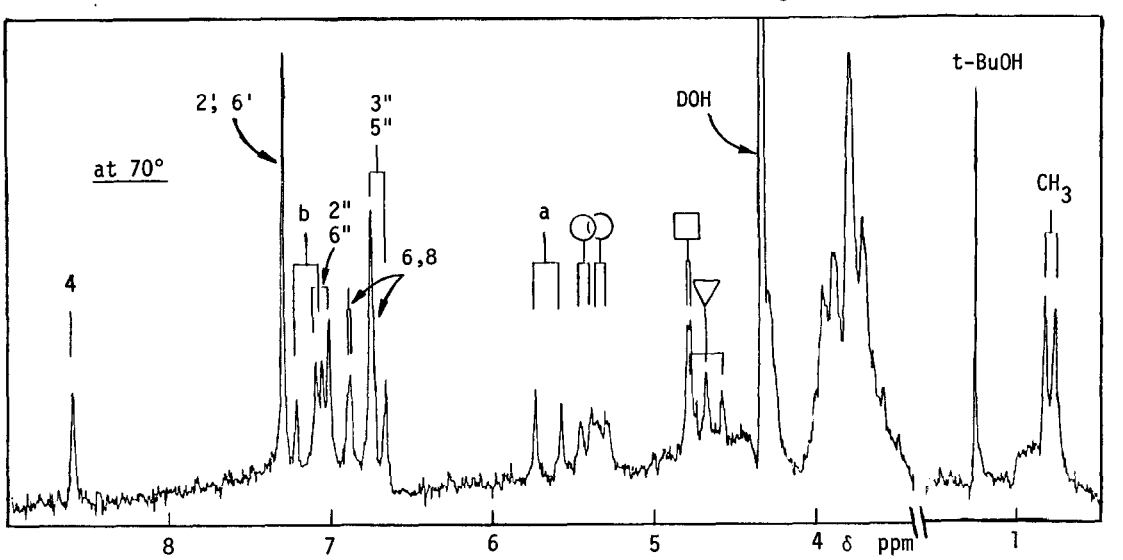


Fig. 2. PMR Spectrum of Violanin Chloride (II) in D_2O

(1H,d,J=16Hz,H_a), 5.48 and 5.17 (each 1H,d,J=7Hz,Glc anomeric H), 4.86 (1H,t,J=9.5Hz,Rha H-4), 4.68 (1H,br.s, Rha anomeric H), 1.00 (3H,d,J=6Hz,Rha CH_3). Analysis: [1], [3] and [4]; similar to the analysis of awobanin, [2] triplet nature of the signal at δ 4.86 indicates the position of attachment of p-coumaroyl group on rhamnose; no other positions can give this signal.

PMR spec. of shisonin chloride (III) (5 mg in 0.3 ml CD_3OD containing 2 μl of 20% DCl in D_2O at 50°) δ 8.87 (1H,s,H-4), 8.14 (1H,q,J=2 and 9Hz,H-6'), 7.90 (1H,d,J=2Hz,H-2'), 7.31 (1H,d, J=16Hz,H_b), 7.18 (2H,$\underline{A}'_2\underline{B}'_2$,$J_{ortho}$=8Hz,H-2" and 6"), 6.92 (1H,d,J=8Hz,H-5'), 6.94 (1H,d,J=ca 2Hz, H-6), 6.86 (1H,d,J=ca 2Hz,H-8),[10] 6.68 (2H,$A'_2\underline{B}'_2$,J_{ortho}=8Hz,H-3" and 5"), 6.12 (1H,d,J=16 Hz, H_a), 5.41 and 5.12 (each 1H,d,J=7Hz,Glc anomeric H), 4.44 (2H,d-like, p-coumaroyl-O$\underline{CH}_2$-). Analysis: [1], [2] and [3]; similar to the analysis of awobanin, and [4] signals of aromatic protons support the cyanidin nucleus.

Deuteriomethanol as solvent gives better spectra than deuterium oxide. Anthocyanins purified by ppc or Avicel tlc (Funakoshi) usually give broader PMR spectra than that purified by Avicel (Merck) column chromatography. The causes are not clear. Violanin purified through its picrate[8] gave the best result. In this case, D_2O solution at an elevated temp. also gave an analyzable PMR spectrum (Fig. 2).

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10. Assignment of H-6 and 8 signals was done by irradiation of H-4 signal; the higher signal become sharper by the irradiation indicating that it corresponds to H-8. In the case of anthocyanidins, this order is reversed.[2]
11. On hydrogen peroxide oxidation followed by hydrolysis violanin gave rutinose (6-O-α-L-rhamnosyl-D-glucose),whose structure was confirmed by comparison of ^{13}C-NMR spectrum with that of an authentic sample (unpublished results).