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Structure of Three Diacylated Anthocyanins Isolated from Red Cabbage, Brassica oleracea

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Three diacylated anthocyanins were isolated from Brassica oleracea and their structures determined to be 3-O-(6-O-acyl-2-O-(2-O-sinapyl-β-D-glucopyranosyl)-β-D-glucopyranosyl)-5-O-(β-D-glucopyranosyl)cyanidins, in which the acyl groups were p-coumaryl, feruloyl, and sinapyl, respectively.

As safe natural food coloring materials, the anthocyanin extracts from red cabbage Brassica oleracea have been widely used. For the color of the extracts is moderately stable in aqueous solutions, predictably in the extracts di or more acylated anthocyanins must exist as the major components.[1,2] Hrazdina and the collaborators[3] had reported the structures of the diacylanthocyanins to be cyanidin 3-diacylglucoside-5-glucosides in which the diacyl groups are di-p-coumaryl, diferuloyl, or disinapyl, but no mixed acyl groups had been found. They assumed that the acylating process in anthocyanin biosynthesis was strictly controlled by enzymes. Recently we have reported the isolation of five monoacyl-anthocyanins from the extract of red cabbage and determined their structures to be 3-O-(6-O-acyl-sophorosyl)-5-O-glucosylcyanidin.[4]

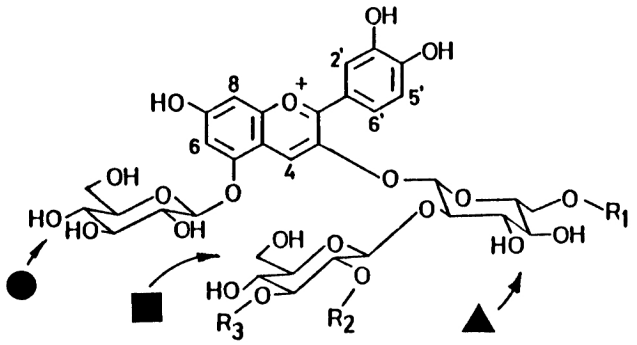
In this paper we wish to report complete stereostructures of three diacylated anthocyanins isolated from B. oleracea.

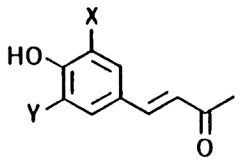
Red cabbage leaves (10 kg) were extracted by the same procedure[4] as reported for the monoacyl anthocyanins. From the extract were obtained crude pigments by Amberlite XAD-7 column chromatography eluted with 60% MeOH containing 1.5% trifluoroacetic acid (TFA). The pigments were separated by repeated ODS-HPLC using 25% solvent A (AcOH:CH_3CN:H_2O=20:25:55) containing 1.5% H_3PO_4 to give a mixture of three diacylanthocyanins (348 mg, chloride form, ratio of **1**:**2**:**3** = 1:7:10), which was further separated by paper chromatography using 6% HCl-H_2O:n-BuOH (4 : 5) to give pure diacylanthocyanins, **1**, **2**, and **3** (chloride form), as dark-red solids.[5,6]

Hydrolysis of **1**, **2**, and **3** was carried out with 2% NaOH at 0 °C in aq. MeOH followed by treatment with 3% TFA in MeOH, and the products were identified in comparison of the t_R (retention time) values with the authentic samples by means of ODS-HPLC. From **1**, **2**, and **3** were produced methyl p-coumarate and sinapate (1:1),

methyl ferulate and sinapate (1:1), and methyl sinapate only, respectively, as well as the same deacylanthocyanin, 3-O-(2-O-(β-D-glucopyranosyl)-β-D-glucopyranosyl)-5-O-(β-D-glucopyranosyl)cyanidin (**4**).[4]

The FABMS of **3** indicated a molecular ion at m/z 1185 (as flavylium ion). ^{1}H NMR of **3** showed the presence of two sinapic acids, three hexosides and cyanidin. By analysis of the difference NOEs by irradiation of the anomeric protons at 5 °C[7] and ^{1}H COSY, all proton signals of **3** could be assigned as shown in Fig. 1. Since irradiation at the anomeric proton of ▲- (5.70), ●- (5.18), and ■-glucose (5.20) caused strong negative NOEs on H-4 (8.81, -25%), H-6 (6.94, -14%, but no NOE on H-8), and ▲-2 (4.14, -8.5%), respectively, the positions of glycosidic linkages were deduced as shown in the formula. All hexoses are β-glucopyranoside form as deduced from the J values ($J_{1,2}$, $J_{2,3}$, $J_{3,4}$, and $J_{4,5}$ = 7.5-9.5 Hz). Two signals corresponding to CH of ■-2 and CH_2 of ▲-6 of **3** were shifted 0.5 - 1 ppm toward lower fields than those of the deacylated anthocyanin **4**. Therefore, the hydroxyl groups at ■-2 and ▲-6 of **3** were acylated with two sinapyl groups. Thus, the structure of **3** was determined to be 3-O-(6-O-sinapyl-2-O-(2-O-sinapyl-β-D-glucopyranosyl)-β-D-glucopyranosyl)-5-O-(β-D-glucopyranosyl)cyanidin.





p-coumaryl X = Y = H
feruloyl X = OCH_3 Y = H
sinapyl X = OCH_3 Y = OCH_3

	R_1	R_2	R_3		R_1	R_2	R_3
1	p-coumaryl	sinapyl	H	**5**	p-coumaryl	H	H
2	feruloyl	sinapyl	H	**6**	H	H	sinapyl
3	sinapyl	sinapyl	H	**7**	p-coumaryl	H	sinapyl
4	H	H	H	**8**	feruloyl	H	H
				9	feruloyl	H	sinapyl

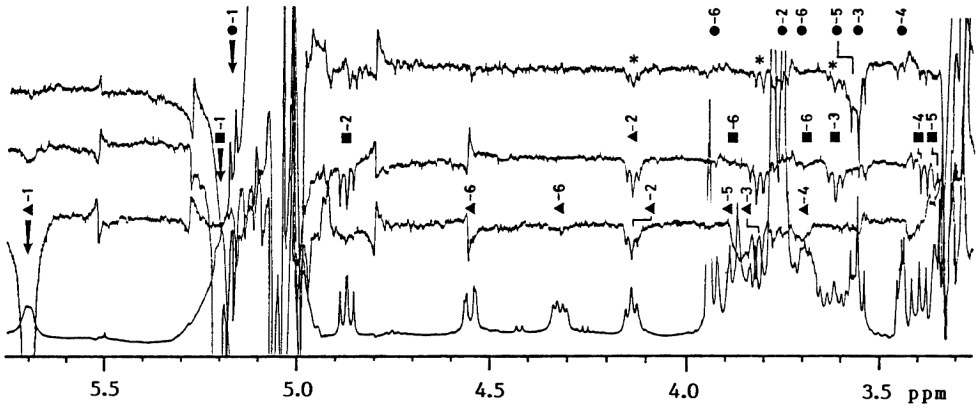


Fig. 1. The difference NOE spectra in the region of the sugar moieties of **3** in 5% TFA-CD_3OD at 5 °C. * : the result of decoupler spillover from ●-1 into ■-1.

By the ^{1}H NMR analysis similar to the case of **3**, the structure of **1** (FABMS; m/z 1125) was determined as a diacylated anthocyanin, involving two alternative acyl groups at 6-OH of ▲ and 2-OH of ■ . Partial hydrolysis of **1** (6 mg) with 3% NaOH in MeOH-H_2O at -19 °C for 80 min followed by treatment with 9% TFA-H_2O gave the products, which were separated by ODS-HPLC using a gradient elution from 40% to 60% solvent A containing 1% TFA to give three anthocyanins, **5** (1.3 mg), **6** (1.1 mg), and **7** (2.6 mg), as well as methyl sinapate and methyl p-coumarate. The pigment **5** was identified to be the monocoumaryl anthocyanin, whose ^{1}H NMR spectrum was superimposable to that of the known ▲-6-O-p-coumarate of **4**.[4] Thus, the structure of **1** was determined to be 3-O-(6-O-p-coumaryl-2-O-(2-O-sinapyl-β-D-glucopyranosyl)-β-D-glucopyranosyl)-5-O-(β-D-glucopyranosyl)cyanidin. The pigment **6** is ■-3-O-sinapate of **4**, and **7** is ■-3-O-sinapyl-▲-6-O-p-coumaryl ester of **4**.[8]

The anthocyain **2** (FABMS; m/z 1155)(7.3 mg) was partially hydrolyzed in the manner similar to the case of **1** to give ▲-6-O-ferulate of **4** (**8**, 0.9 mg),[4] **6** (1.3 mg), and ■-3-O-sinapyl-▲-6-O-feruly1 ester of **4** (**9**, 1.8 mg).[9]

Three diacylanthocyanins, **1**, **2**, and **3**, are fairly stable in neutral aq. solutions, and the monoacylanthocyanin, **5**, slightly unstable, whereas **4** is quickly decolorized. At pH 6.5 the λ_{max} of three diacylates are shifted ca 40 nm toward longer wavelengths in comparison with that of **4**. These results indicate that in the diacylated anthocyanins in anhydrobase form the aromatic acyl groups stack on and under the anthocyanidin nucleus in a sandwich type.

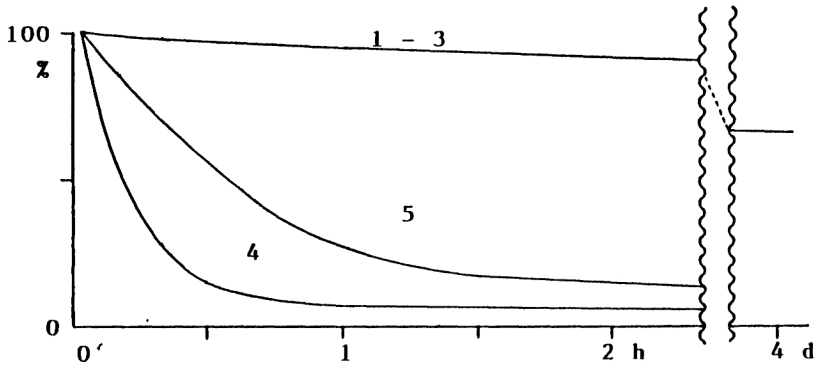


Fig. 2. Stability of anthocyanins (**1-3**, **5**, and **4**) in 1/15 mol dm^{-3} phosphate buffer solution at pH 6.5 (concd 3.3 X 10^{-5} mol dm^{-3} ; observed at λ_{max} of visible absorption).

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References

1) I. Chmielewska, I. Kakowska, and B. Lipinski, Bull. Acad. Pol. CI.III, 3, 527 (1955).
2) J. B. Harborne, Phytochem., 3, 151 (1964).
3) G. Hrazdina, H. Iredale, and L. R. Mattick, Phytochem., 16, 297 (1977).
4) E. Idaka, K. Suzuki, H. Yamakita, T. Ogawa, T. Kondo, and T. Goto, Chem. Lett., 1987, 145.

5) In order to obtain an anthocyanin chloride in a solid state, the following procedure was performed. The eluate was poured on an Amberlite XAD-7 column, which was washed with 1.5% TFA, and then eluted with solvent A containing 1.5% TFA. The eluates containing pigments were dried in vacuo, the pigments were dissolved in 1% HCl-MeOH and precipitated by addition of Et_2O to give a pure anthocyanin chloride as a dark-red solid.

6) Spectral data of three anthocyanins (**1** - **3**).

	1	**2**	**3**
UV (1%HCl) λ_{nm} (ε x10^3)	537(19)	539(22)	540(17)
	321(19)	332(23)	332(19)
	294(19)	295(20)	298(15)
$E_{UV\ max}/E_{VIS\ max}$	1.01	1.04	1.11

^{1}H NMR (10% CF_3COOD-CD_3OD, at 20 °C, δ ppm)

	1	**2**	**3**
▲-1	5.73	5.69	5.67
▲-2	4.11	4.13	4.12
▲-3	3.85	3.83	3.81
▲-4	3.59	3.64	3.67
▲-5	3.89	3.87	3.85
▲-6	4.46	4.50	4.55
▲-6	4.39	4.36	4.32
●-1	5.17	5.16	5.15
●-2	3.78	3.76	3.76
●-3	3.59	3.57	3.56
●-4	3.48	3.46	3.45
●-5	3.61	3.58	3.56
●-6	3.99	3.95	3.94
●-6	3.75	3.72	3.70
■-1	5.22	5.23	5.17
■-2	4.89	4.89	4.7
■-3	3.64	3.64	3.61
■-4	3.43	3.42	3.40
■-5	3.37	3.36	3.35
■-6	3.89	3.89	3.87
■-6	3.69	3.69	3.66

	1	**2**	**3**
H-4[a)]	8.81	8.81	8.85
H-6	6.95	6.94	6.95
H-8	6.66	6.65	6.65
H-2'	7.85	7.83	7.85
H-5'	7.08	7.06	7.07
H-6'	8.21	8.24	8.27
H-α[b)]	6.13 6.17	6.12 6.20	6.23 6.12
H-β	7.28 7.35	7.27 7.35	7.41 7.26
H-2	7.22 6.58	6.85 6.59	6.65 6.59
H-3	6.75 -	- -	- -
H-5	6.75 -	6.73 -	- -
H-6	7.22 6.58	6.83 6.59	6.65 6.59
CH_3O	- 3.78	3.79 3.78	3.80 3.79

J values in glucose moieties

	$J_{1,2}$	$J_{2,3}$	$J_{3,4}$	$J_{4,5}$	$J_{5,6}$	$J_{5,6}$	$J_{6,6}$
(Hz)	7.5	9.0	9.5	9.5	6.5	2.0	12.5

	NOE (%) ▲-1→H-4	●-1→H-6	■-1→▲-2
1(20 °C)	-6.5	-2.0	-
2(20 °C)	-12.5	-6.0	-3.5
3(5 °C)	-25.0	-14.0	-8.5

a; cyanidin nuclei
b; aromatic acid moieties

7) T. Kondo, H. Tamura, T. Kawai, and T. Goto, Tetrahedron Lett., 28, (1987) in press.

8) Since the proton at 4.96 ppm (triplet, J=9.5 Hz) is attributable to ■-3, while the proton of ■-2 appears at 3.3 ppm as determined by spin-decoupling experiments at 0 °C, and since signals corresponding to a p-coumaryl group were lost in the aromatic region of ^{1}H NMR by comparison with that of **1**, the structure of **6** was elucidated to be the ■-3-O-sinapate of **4**. The protons of **7** (5.03, and 4.51 and 4.45) corresponding to ■-3 and ▲-6, respectively, were shifted toward downfields more than 0.5 ppm in comparison with that of **4**. Therefore the structure of **7** is 3-O-(6-O-p-coumaryl-2-O-(3-O-sinapyl-β-D-glucopyranosyl)-β-D-glucopyranosyl)-5-O-(β-D-glucopyranosyl)cyanidin. In the alkaline solution the migration of the acyl group from the 2-OH to the 3-OH predominated over the hydrolysis.

9) In the hydrolysis of **3** the results similar to **2** were obtained.

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