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A UV-B resistant polyacylated anthocyanin, HBA, from blue petals of morning glory

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Abstract—Prevention of *E*,*Z*-isomerization of caffeoyl residues of a tri-(*E*)-caffeoyl anthocyanin, heavenly blue anthocyanin (HBA), and its stability under UV-B irradiation conditions were studied. We isolated four photoproducts from irradiated HBA and their structures were determined to be mono- or di-*Z*-caffeoyl isomers of HBA and mono-deglucosylated HBA. Under such conditions one caffeoyl residue, the innermost one, never isomerized to the *Z*-form, suggesting that intramolecular stacking must prevent photoisomerization. In general, anthocyanins are considered to be more stable in strong acidic than neutral aqueous media. However, with UV-B irradiation HBA was most stable in aqueous solution at pH 7.5 and most unstable in acidic methanol solutions. It was found to emit strong fluorescence on excitation with UV-B, possibly resulting from intramolecular association of caffeoyl moieties with the anthocyanidin nucleus. The finding that pigment in petals is more tolerant of UV-irradiation may be rational in the context of the necessity to resist strong solar radiation.

Plants grow and reproduce under exposure to strong sunlight, which contains harmful UV rays causing DNA damage and lipid peroxidation.[1] Flavonoids, essential in higher plants, play an important role in absorbing and screening against UV.[2] Polyacylated anthocyanins[3] which possess two or more cinnamoyl derivative residues (*p*-coumaroyl, caffeoyl, and feruloyl moieties) show strong absorbance peaks in the UV-B region and are also expected to be protective.[2c,4] Recently we encountered an interesting phenomenon; α,β double bonds of caffeoyl moieties in diacylated anthocyanins hardly isomerize to *Z*-forms,[5] though, simple cinnamoyl derivative esters and such residues in monoacylated anthocyanins readily isomerize on UV-B irradiation.[6] In this report we describe the prevention of *E*,*Z*-isomerization and unexpected stability of a tri-caffeoyl anthocyanin, heavenly blue anthocyanin (HBA),[7] from blue petals of *Ipomoea tricolor* cv. Heavenly blue under physiological conditions. The mechanism of UV tolerance and stability with regard to molecular stacking and energy quenching is discussed.

HBA (**1**) was dissolved in a 0.1% HCl–methanol solution (5×10^{-5} M) and irradiated with a high-pressure mercury arc lamp for 10 min.[6c,8] On analysis of reversed phase HPLC,[9] four new peaks were observed (Fig. 1). From the irradiated solution of **1** (245 mg in 0.5%

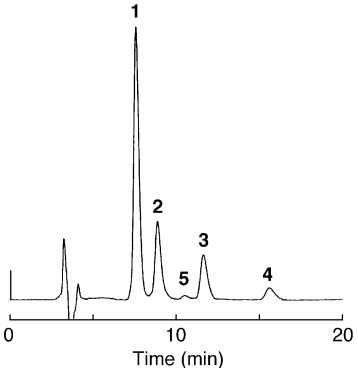

Figure 1. HPLC chromatogram of UV-B irradiated HBA (**1**) in 0.1% HCl–MeOH (5×10^{-5} M, 25°C, 10 min).

Keywords: heavenly blue anthocyanin; polyacylated anthocyanin; intramolecular stacking; *E*,*Z*-isomerization; UV light tolerance; caffeoyl residue.

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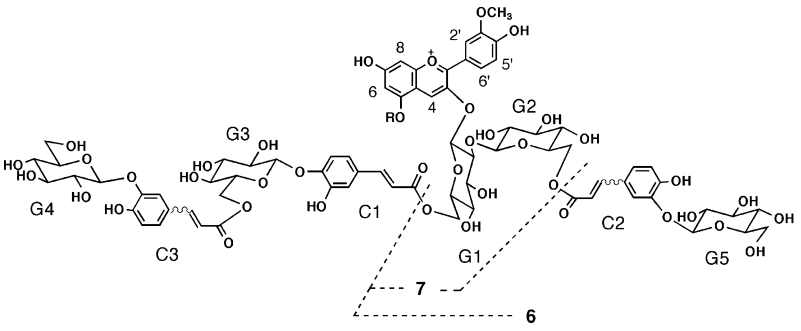

	R	C1	C2	C3
HBA (**1**)	β-Glc (G6)	*E*	*E*	*E*
2	β-Glc (G6)	*E*	*Z*	*E*
3	β-Glc (G6)	*E*	*E*	*Z*
4	β-Glc (G6)	*E*	*Z*	*Z*
5	H	*E*	*E*	*E*
6	β-Glc (G6)	—	*E*	—
7	β-Glc (G6)	—	—	—

Scheme 1.

TFA–MeOH, high pressure mercury arc, at 0°C for 50 min)[5,6c] **1** (168 mg, 69%), **2** (21 mg, 8.7%), **3** (16 mg, 6.7%), and **4** (1.7 mg, 0.7%) were purified with preparative HPLC (Develosil® ODS-HG-5, 20 mm ϕ×250 mm) by stepwise elution from 0 to 20% aq. CH_3CN containing 0.5% TFA as a dark red TFA salt. In acidic solutions the photoproduct **5** yield was very low. Therefore **1** was irradiated with UV-A (380 mg in 100 mM acetate buffer at pH 5.0, at 25°C for 2 h)[10] and purified to give **5** (34 mg, 9.9%) and **1** (107 mg, 28%) with the same ODS-HPLC system.

FABMS of **2**, **3** and **4** with 1N HCl-glycerol as a matrix showed the same molecular ion peak at $m/z=1759$ [M^+] as **1**, indicating the compounds to be isomers of HBA. FABMS of **5** showed the molecular ion peak at $m/z=$ 1597 [M^+], attributable to loss of one glucosyl residue from **1**. With various 1D and 2D NMR experiments the structures of **2**, **3** and **4** were determined to be isomers of HBA in which one or two caffeoyl residues were isomerized to the *Z*-form. The structure of **5** was elucidated to be 5-deglucosyl HBA (Scheme 1, Table 1).[11] Under the irradiation conditions the inner caffeoyl residue, C1, never isomerized to a *Z*-form.

Although we could obtain isomerized photoproducts of HBA by UV irradiation in vitro, these isomers could not be observed in living flower petals of morning glory. Therefore, there must be some mechanism preventing of isomerization in vivo. In flower petals anthocyanins are located in central vacuoles. In blue morning glory, the vacuolar pH increases from 6.6 to 7.6 during blooming.[12] *E*,*Z*-isomerization and stability of HBA with UV-B irradiation was examined in aqueous solutions at various pH values. HBA or other photoproducts (**2**, **3** and **4**) were dissolved in acidic methanol, acidic water, buffer solutions at pH 4.0, 6.0, and 7.5 with 5×10^{-5} M, poured into a quartz cuvette and irradiated with broad UV-B light from a high-pressure mercury arc.[6c,8] HPLC analysis showed that within 5 min the photoisomerization reached equilibrium; in a 0.1% HCl–methanol solution the ratios for **1**, **2**, **3**, and **4** were 63:20:12:5, while in aqueous solution at pH 7.5 that were 97:1:2:0, indicating that in neutral aqueous solution photoisomerization was more depressed. Methyl caffeate in acidic methanol and pH 7.5 buffer gave equilibrium mixture of 55:45 (*E*/*Z*) and 95:5, respectively. Addition of tris-deacyl HBA (**7**)[13] gave no difference on the ratio. Therefore, intramolecular caffeoyl residues must be affected. Several pieces of experimental evidence support an intramolecular stacking conformation of **1**, with reference to increase of stability, high-field shift of aromatic protons in ^{1}H NMR, and long range NOEs.[3a,c,14,15] Therefore, there must exist the same preventing mechanism as for gentiodelphin in the *E*,*Z*-isomerization reaction of caffeoyl moieties of **1** due to intramolecular stacking to the anthocyanidin nucleus.[5]

In order to study UV tolerance, **1** was irradiated for a longer time in various solutions. In general, anthocyanins are most stable under strong acidic conditions and become unstable with increase in the pH of the solution.[3,16] HBA solution also showed the same behavior in the dark. However, under UV irradiation **1** was most stable in aqueous media at pH 7.5 and most unstable in acidic methanol (Fig. 2).

In aqueous solutions at any pH, bis-isomerized products (**4**) was not detected after 15 h irradiation, while

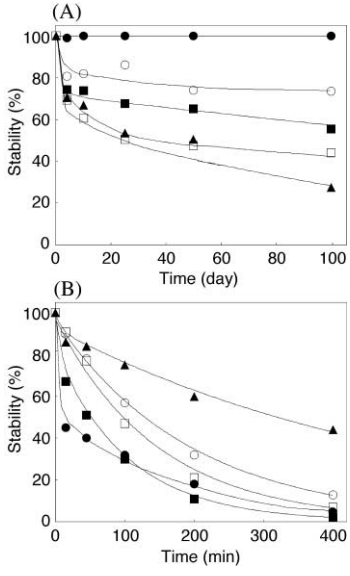

Figure 2. Stability of HBA (**1**) in the dark (A) and under UV-B irradiated (B)[8] conditions in various solutions (5×10^{-5} M at 25°C) measured by HPLC. ●: 0.5% TFA–MeOH, ○: 0.5%TFA–H_2O, ■: acetate buffer pH 4.0, □: phosphate buffer pH 6.0, ▲: phosphate buffer pH 7.5.

Table 1. ^{1}H NMR data for photoproducts of HBA (**2**, **3**, **4** and **5**) in 5% TFA*d*-CD_3OD[a]

Position		**2**			**3**			**4**			**5**		
		(ppm)	(mult., *J*, Hz)		(ppm)	(mult., *J*, Hz)		(ppm)	(mult., *J*, Hz)		(ppm)	(mult., *J*, Hz)	
4		8.75	s		8.92	s		8.78	s		8.91	s	
6		6.83	d	2.0	6.80	d	2.0	6.82	d	2.0	6.43	br. s	
8		6.49	d	2.0	6.67	d	2.0	6.66	d	2.0	6.25	br. s	
2′		7.56	br. s		7.86	d	2.0	7.75	d	2.0	7.58	d	2.0
5′		6.93	d	9.0	6.97	d	8.5	6.98	d	8.5	6.85	d	8.5
6′		8.30	dd	9.0, 2.0	8.28	dd	8.5, 2.0	8.36	dd	8.5, 2.0	8.18	dd	8.5 2.0
OCH_3		3.89	s		3.91	s		3.96	s		3.75	s	
G1	1	5.30	d	7.0	5.38	d	7.0	5.28	d	7.0	5.33	d	7.5
	2	3.90	dd	9.0, 7.0	3.95	dd	9.0, 7.0	3.92	dd	9.0, 7.0	3.92	dd	9.0, 7.5
	3	3.77	t	9.0	3.78	br. dd	9.5, 9.0	3.76	br. dd	9.5, 9.0	3.77	t	9.0
	4	3.51	dd	9.5, 9.0	3.58	t	9.5	3.56	t	9.5	3.51	t	9.0
	5	3.83	ddd	9.5, 8.5, 2.5	3.73	ddd	9.5, 7.0, 2.0	3.68	ddd	9.5, 7.0, 2.5	3.85	br. dt	9.0, 2.0
	6a	4.37	dd	12.0, 2.5	4.34	dd	12.0, 7.0	4.36	dd	12.0, 7.0	4.30	dd	12.0, 2.0
	6b	4.51	dd	12.0, 8.5	4.43	dd	12.0, 2.0	4.45	dd	12.0, 2.5	4.49	dd	12.0, 8.5
G2	1	4.74	d	7.5	4.75	d	7.0	4.74	d	7.5	4.74	d	7.5
	2	3.47	m		3.43	m		3.46	m		3.39	dd	9.0, 7.5
	3	3.47	m		3.43	m		3.46	m		3.47	t	9.0
	4	3.39	m		3.43	m		3.36	br. t	9.0	3.31	dd	9.5, 9.0
	5	3.52	m		3.43	m		3.53	ddd	9.0, 6.5, 2.5	3.56	ddd	9.5, 7.0, 2.0
	6a	4.31	dd	12.0, 2.5	4.12	br. d	11.5	4.29	dd	12.0, 2.5	4.14	dd	11.5, 7.0
	6b	4.42	dd	12.0, 7.0	4.27	br. d	11.5	4.43	dd	12.0, 6.5	4.26	dd	11.5, 2.0
G3	1	4.93	d	7.5	4.88	d	7.0	4.87	d	7.0	4.94	d	7.5
	2	3.60	dd	9.0, 7.5	3.57	dd	9.0, 7.0	3.58	dd	9.0, 7.0	3.60	dd	9.0, 7.5
	3	3.54	t	9.0	3.53	t	9.0	3.53	t	9.0	3.54	t	9.0
	4	3.41	dd	9.5, 9.0	3.39	dd	9.5, 9.0	3.40	dd	9.5, 9.0	3.39	br. dd	9.0, 8.5
	5	3.77	ddd	9.5, 8.5, 2.0	3.73	ddd	9.5, 7.5, 2.0	3.72	ddd	9.5, 7.5, 2.5	3.74	td	8.5, 2.0
	6a	4.25	dd	11.5, 8.5	4.43	dd	12.0, 2.0	4.43	dd	12.0, 2.5	4.19	dd	11.5, 8.5
	6b	4.73	dd	11.5, 2.0	4.50	dd	12.0, 7.5	4.51	dd	12.0, 7.5	4.80	dd	11.5, 2.0
G4	1	4.61	d	7.5	4.48	d	7.5	4.51	d	7.5	4.49	d	7.0
	2	3.47	m		3.44	dd	9.0, 7.5	3.46	m		3.47	m	
	3	3.47	m		3.49	m		3.47–3.52	m		3.47	m	
	4	3.38	m		3.49	m		3.47–3.52	m		3.36	br. t	9.0
	5	3.42	m		3.42	m		3.44	m		3.41	ddd	9.0, 7.5, 2.5
	6a	3.68	dd	12.0, 6.0	3.79	dd	12.0, 4.5	3.78	dd	12.0, 7.5	3.66	dd	12.0, 7.5
	6b	3.91	dd	12.0, 2.5	4.01	dd	12.0, 2.0	4.01	dd	12.0, 2.0	3.91	dd	12.0, 2.5
G5	1	4.52	d	7.5	4.78	d	7.5	4.59	d	7.5	4.66	d	7.5
	2	3.41	m		3.52	m		3.44	dd	9.0, 7.5	3.49	m	
	3	3.45–3.53	m		3.52	m		3.43–3.54	m		3.49	m	
	4	3.45–3.53	m		3.39	t	9.0	3.43–3.54	m		3.36	br. t	9.0
	5	3.38	m		3.44	ddd	9.0, 6.0, 2.5	3.42	m		3.40	ddd	9.0, 7.5, 2.0
	6a	3.76	m		3.67	dd	12.0, 6.0	3.78	dd	12.0, 7.0	3.65	dd	12.0, 7.5
	6b	3.89	m		3.91	dd	12.0, 2.5	3.91	dd	12.0, 2.5	3.89	dd	12.0, 2.0

Table 1. ^{1}H NMR data for photoproducts of HBA (**2**, **3**, **4** and **5**) in 5% TFA*d*-CD_3OD[a]

Position		**2**			**3**			**4**			**5**		
		(ppm)	(mult., *J*, Hz)		(ppm)	(mult., *J*, Hz)		(ppm)	(mult., *J*, Hz)		(ppm)	(mult., *J*, Hz)	
G6	1	5.21	d	7.5	5.03	d	7.5	5.20	d	7.5			
	2	3.76	dd	9.0, 7.5	3.70	dd	9.0, 7.5	3.76	dd	9.0, 7.5			
	3	3.63	t	9.0	3.55	t	9.0	3.61	t	9.0			
	4	3.51	t	9.0	3.45	t	9.0	3.52	t	9.0			
	5	3.62	ddd	9.0, 6.0, 2.0	3.51	ddd	9.0, 7.0, 2.0	3.59	ddd	9.0, 6.0, 2.0			
	6a	3.81	dd	12.0, 6.0	3.75	dd	12.0, 7.0	3.81	dd	12.0, 6.0			
	6b	4.03	dd	12.0, 2.0	3.97	dd	12.0, 2.0	4.02	dd	12.0, 2.0			
C1	α	5.85	d	16.0	5.84	d	16.0	5.90	d	16.0	5.81	d	16.0
	β	7.10	d	16.0	7.04	d	16.0	7.05	d	16.0	7.13	d	16.0
	2	6.88	d	2.0	6.79	d	2.0	6.82	d	2.0	6.88	d	2.0
	5	7.06	d	8.5	6.94	d	8.5	6.95	d	8.5	7.05	d	8.5
	6	6.63	dd	8.5, 2.0	6.52	dd	8.5, 2.0	6.55	dd	8.5 2.0	6.70	dd	8.5, 2.0
C2	α	5.24	d	13.0	5.78	d	16.0	5.25	d	13.0	5.63	d	16.0
	β	6.16	d	13.0	6.96	d	16.0	6.21	d	13.0	6.89	d	16.0
	2	7.75	d	2.0	7.11	br. s		7.75	d	2.0	7.04	d	2.0
	5	6.47	d	8.5	6.71	br. s		6.46	d	8.5	6.59	br. s	
	6	6.63	dd	8.5, 2.0	6.71	br. s		6.66	dd	8.5, 2.0	6.59	br. s	
C3	α	6.30	d	16.0	5.79	d	13.0	5.81	d	13.0	6.24	d	16.0
	β	7.45	d	16.0	6.73	d	13.0	6.77	d	13.0	7.41	d	16.0
	2	7.41	d	2.0	7.95	d	2.0	7.96	d	2.0	7.29	d	2.0
	5	6.51	d	8.5	6.67	d	8.5	6.70	d	8.5	6.48	d	8.5
	6	6.69	dd	8.5, 2.0	6.98	dd	8.5, 2.0	7.03	dd	8.5, 2.0	6.61	dd	8.5, 2.0

[a] NMR spectra were measured with ECA-500 (^{1}H: 500 MHz) at rt.

bleaching of pigments progressed. Photo-deglucosylation reaction occurred under weakly acidic conditions and under strong acidic or weakly alkaline solutions **5** was present as a trace. The reason why HBA is more tolerant to UV under physiological conditions is unknown, therefore, we search for light energy quenching system in **1**. When **1** in aqueous solution at pH 7.5 was irradiated with UV-B light, strong red fluorescence of λmax at 652 nm was observed. However, at lower pH the fluorescence intensity decreased and in strong acidic methanol and acidic water solutions almost no fluorescence was emitted (Fig. 3). Deacylated pigments, bis-deacyl HBA (**6**)[17] and tris-deacyl HBA (**7**)[13] in aqueous solutions at pH 7.5 emit very little fluorescence; the intensity of **6** and **7** was 1/5 and 1/10, respectively, as compared with **1**. Addition of methyl caffeate to aqueous solutions of **6** or **7** at pH 7.5 (3 and 30 equiv.) did not affect the intensity of the fluorescence, suggesting that intramolecular caffeoyl moieties play some important role.[18]

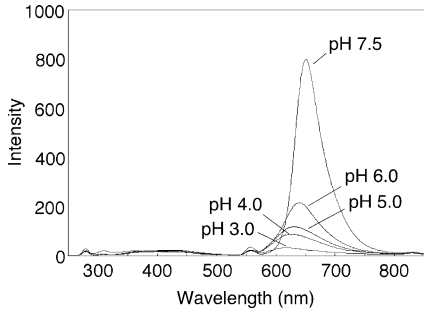

Figure 3. Fluorescence spectra of HBA (**1**) in various aqueous solutions excited at 280 nm (5×10^{-5} M, 25°C).

In conclusion, molecular associations in HBA must prevent the *E*,*Z*-isomerization reaction. The light energy absorbed with caffeoyl residues might be transferred to the anthocyanidin nucleus to emit red fluorescence. These mechanisms may work efficiently in morning glory petals and this could be the reason why polyacylated anthocyanins do not isomerize to the *Z*-form but rather are tolerant of UV-B. To protect DNA by screening against UV-B irradiation may be one of the most important functions of anthocyanins in living petal cells and rational in terms of plant survival.

Acknowledgements

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8. An Eikosha's high-pressure mercury arc, EHB-WU-100, 100 W, radiating from 250 to 600 nm, but mainly at 365 nm, was used. The radiation energy was controlled at 5000 μw/cm^2 measured using a MINOLTA's UV radiometer UM-10 and a UM-360 receptor with cut off of waves shorter than 290 nm with a UV-29 cut filter (Toshiba Glass). Light transparency at 260 nm, 290 nm and 340 nm was 10%, 50% and 90%, respectively.
9. Develosil ODS-HG-5 column (2.0 mm ϕ×250 mm) with 18% aq. CH_3CN containing 0.5% TFA as the eluent, flow rate: 0.2 mL/min, detection: 280 nm, temperature: 40°C.
10. The sample solution was irradiated with the same mercury arc through a 10 mm path filter of 100 mM $CuSO_4$. The light energy through the filter was 1100 μw/cm^2 with the UM-250 and 31000 μw/cm^2 with the UM-360 receptor.
11. **2**: UV/vis (0. 1% HCl–MeOH) nm (ε): 289 (32,000), 317 (29,000), 530 (23,000), **3**: UV/vis (0.1% HCl–MeOH) nm (ε): 295 (35,000), 319 (23,000), 533 (24,000), **4**: UV/vis (0.1% HCl–MeOH) nm (ε): 285 (33,000), 320 (30,000), 528 (24,000), **5**: UV/vis (0.1% HCl–MeOH) nm (ε): 289 (34,000), 316 (28,000), 533 (19,000).
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15. In 5%TFA*d*–CD_3OD the most proton signals of C1–C3 in **1**–**5** were shifted toward higher field (ca. 0.2–0.65 ppm)

by comparison with those of methyl (*E*)-caffeate (ppm α: 6.25, β: 7.53, 2: 7.02, 5: 6.77, 6: 6.93) or methyl (*Z*)-caffeate (α: 5.73, β: 6.77, 2: 7.34, 5: 6.72, 6: 7.03). Long range NOEs were observed between the following protons of **1**: H4 and C1α, C1β, C2α, C2β, G2-1, G2-6, H6 and G4-1, H8 and C2α, C2β, C3α, C3β, G4-1, G5-1, 2′ and C3-5, 5′ and G2-6, 6′ and G2-1, C3-5, -OCH_3 and C2-5. Using those NMR data and the calculation of the ratio of rotamers of *exo* cyclic C5–C6 bonds of glucosyl residues (G1–G6),[19] molecular modeling was performed. Preliminary intramolecular stacking structure in which C1 stacked to one side of the peonidin nucleus, C2 and C3 did to the other side was obtained.

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18. Gentiodelphin[14a] from blue petals of *Gentiana triflora* emitted fluorescence of λmax at 621 nm by UV-B irradiation under the same condition (5×10^{-5} M at pH 7.5). In acidic aqueous solution no fluorescence was observed.
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