fragment. The chemical shifts were typical for methylene in an aliphatic chain (1.5 ppm) and methylene substituted by an aromatic ring (2.5 ppm). In MeOH-d_4 this ethane fragment probably has an exclusively *trans* arrangement of substituents since solvation of the C-7 and C-3″ hydroxyl groups will destroy any hydrogen bonding between these OH groups; such hydrogen bonding is the only factor which could offset the normal steric repulsions which reduce the population of the *gauche* conformer. A *trans* conformation about the bond between C-1″ and C-2″ implies a molecular shape for kievitone hydrate closely similar to that of its precursor kievitone. The nonequivalent Me groups of kievitone (**1**) were replaced by a 6-proton singlet in the spectrum of (**2**), indicating that hydration of the C=C group of kievitone had occurred in a Markovnikov fashion to yield a 3″-OH rather than a 2″-OH side chain. The presumably enzymatically-catalysed hydration was highly specific since no evidence of the system with a 2″-OH side chain was found in the spectrum.

Table 1. Chemical shifts (ppm) of kievitone and kievitone hydrate

Proton	Kievitone hydrate		Kievitone [9]
	MeOH-d_4	DMSO-d_6	DMSO-d_6/$CDCl_3$
H-6	5.95 (*s*)	5.98 (*s*)	6.04 (*s*)
H-1″	2.8 (*m*)	2.5 (*m*)	3.21 (*d*)
H-2″	1.6 (*m*)	1.5 (*m*)	5.18 (*t*)
H-4″	1.25 (*s*)	1.15 (*s*)	1.64 (*s*)
Me-3″			1.74 (*s*)
H-2a	4.5 (*m*)	4.45 (*m*)	4.49 (*m*)
H-2b			4.64 (*m*)
H-3	4.15 (*dd*)	4.15 (*dd*)	4.16 (*dd*)
H-3′	6.31 (*d*)	6.3 (*d*)	6.45 (*d*)
H-5′	6.25 (*dd*)	6.15 (*dd*)	6.32 (*dd*)
H-6′	6.97 (*d*)	6.78 (*d*)	6.91 (*d*)

In MeOH-d_4 all OH protons appeared as a single peak at 4.7 ppm due to rapid exchange. Some exchange was also evident in DMSO-d_6 since the phenolic protons for the 7-OH, 2′-OH and 4′-OH groups appeared as a very broad peak at *ca* 9.5 ppm. The aliphatic tertiary OH group was present as a very broad absorption around 3.8 ppm. A sharp singlet at 12.3 ppm (compared with 12.2 ppm in kievitone) confirmed the presence of the strongly intramolecular-hydrogen-bonded OH at C-5.

Preliminary bioassays of kievitone hydrate against *Cladosporium herbarum* (Pers.) Link. [10], *Aphanomyces euteiches* Drechs. and *Rhizoctonia solani* Kühn indicated that this metabolite was considerably less fungitoxic than kievitone. The metabolic conversion of kievitone to kievitone hydrate by *F. solani* f.sp. *phaseoli*, therefore, may well explain the comparative insensitivity of this fungus to the phytoalexin.

EXPERIMENTAL

Kievitone was obtained following procedures outlined previously [2]. Actively growing mycelium of *F. solani* f.sp. *phaseoli* was obtained as described elsewhere [5]. Kievitone dissolved in EtOH was added to fungal cultures at concns of 25–50 μg/ml; the final EtOH conc in the medium was 0.5%. The cultures were incubated at 25° on a reciprocal shaker for 6 hr, after which kievitone hydrate was isolated by slight modification of methods reported elsewhere [5]. The metabolite was purified by sequential TLC on Si gel G developed with C_6H_6–MeOH (9:1) (R_f 0.16) and toluene–ethyl formate–HCO_2H (7:2:1) (R_f 0.07). Further purification was obtained by gel filtration through Sephadex LH-20 employing EtOH as the eluant. Bioassays were carried out as described in refs. [2, 10].

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ANTHOCYANIN COMPOSITION OF *BRASSICA OLERACEA* CV. RED DANISH

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Key Word Index—*Brassica oleracea* cv. Red Danish; Cruciferae; anthocyanins; malonyl-, *p*-coumaryl-, ferulyl-, sinapyl esters of cyanidin-3-sophoroside-5-glucoside.

Abstract—Eight anthocyanins were isolated from illuminated red cabbage seedlings. They were identified as: cyanidin-3-sophoroside-5-glucoside, cyanidin-3-malonyl-sophoroside-5-glucoside, cyanidin-3-*p*-coumaryl-sophoroside-5-glucoside, cyanidin-3-(di-*p*-coumaryl)sophoroside-5-glucoside, cyanidin-3-ferulyl-sophoroside-5-glucoside, cyanidin-3-(diferulyl)sophoroside-5-glucoside, cyanidin-3-sinapylsophoroside-5-glucoside, and cyanidin-3-(disinapyl)-sophoroside-5-glucoside.

INTRODUCTION

The basic anthocyanin component of red cabbage has been established as the cyanidin-3-sophoroside-5-glucoside [1, 2]. This is present in the plant in combination with *p*-coumaric and ferulic (1) and/or sinapic acids [2, 3]. The existing controversy on the nature of acyl groups of the red cabbage pigments prompted us to reinvestigate the anthocyanin content of this plant.

RESULTS

Column chromatography of the extract from 6-day-old red cabbage seedlings on PVP separated 5 anthocyanin-containing bands. The first two of these bands migrated as single spots on cellulose TLC, while the remaining three bands each separated into two components. Using repeated column chromatography, the first two anthocyanins were obtained pure. Preparative PC of the remaining three fractions yielded a further six anthocyanins. Alkaline hydrolysis did not result in any change in the chromatographic and spectral properties of pigment 1, identified as the cyanidin-3-sophoroside-5-glucoside (Table 1). The other seven pigments only produced cyanidin-3-sophoroside-5-glucoside after treatment with KOH and were all acylated. An aliphatic acid was a component of pigment 2, and sinapic, ferulic, and *p*-coumaric acids were components of pigments 3a and 3b, 4a and 4b, and 5a and 5b, respectively. The aliphatic acid of pigment 2 was identified by GLC as malonic acid, establishing the structure of pigment 2 as cyanidin-3-malonylsophoroside-5-glucoside. The chromatographic and spectral properties (Table 1) of the other six anthocyanins identified them as cyanidin-3-sinapylsophoroside-5-glucoside (3a), cyanidin-3-disinapylsophoroside-5-glucoside (3b), cyanidin-3-ferulylsophoroside-5-glucoside (4a), cyanidin-3-diferulylsophoroside-5-glucoside (4b), cyanidin-3-*p*-coumarylsophoroside-5-glucoside (5a) and cyanidin-3-(di-*p*-coumaryl)sophoroside-5-glucoside (5b).

The pigments of *Brassica oleracea* cv. Red Danish could only be separated into 3 components prior to column chromatography on PVP, the three components showing close similarities to rubrobrassicins A, B, and C (1), respectively. All three rubrobrassicins are thus mixtures. Rubrobrassicin A is composed of cyanidin-3-*p*-coumarylsophoroside-5-glucoside and cyanidin-3-(di-*p*-coumaryl)-sophoroside-5-glucoside. Rubrobrassicin B is a mixture of cyanidin-3-feruylsophoroside-5-glucoside and cyanidin-3-(diferulyl)-sophoroside-5-glucoside. Rubrobrassicin C has 3 components; cyanidin-3-sinapylsophoroside-5-glucoside, cyanidin-3-(disinapyl)sophoroside-5-glucoside and cyanidin-3-malonylsophoroside-5-glucoside. When cyanidin-3-*p*-coumarylsophoroside-5-glucoside was subjected to Dowex hydrolysis (4), 6-0-*p*-coumarylglucose and a second UV-fluorescent compound (R_f 0.46 and 0.51 in solvents 1 & 2) was formed, the latter giving a spot identical to that of 6-0-*p*-coumarylglucose on further hydrolysis. Similarly, Dowex hydrolysis of cyanidin-3-(di-*p*-coumaryl)-sophoroside-5-glucoside yielded 6-0-*p*-coumarylglucose and another UV-fluorescing compound (R_f 0.62 and 0.71 in solvents 1 & 2), which was further hydrolyzed to 6-0-*p*-coumarylglucose. From these data it seems that acylation in anthocyanins *de rigor* occurs at the 6-OH group of the glucose or glucoses attached at the 3-position of the pigment. Lacking authentic compounds, the position of acylation of other pigments in *Brassica* could not be established, but since the sugar moieties in the 3-positions are identical, it seems likely that the position of acylation is the same as in the *p*-coumaryl derivatives. Since no mixed acylated anthocyanins, e.g. where *p*-coumaric and ferulic acids are present in the same molecule, a strict enzymatic control of biosynthesis steps is apparent. Therefore *Brassica oleracea* cv. Red Danish seems to be an ideal model system for investigations on the mechanisms of anthocyanin acylation.

EXPERIMENTAL

Plant material. The anthocyanins were isolated from 6-day-old illuminated seedlings of *Brassica oleracea* cv. Red Danish.

Isolation of pigments. 70 g *Brassica* seedlings were extracted

Table 1. Spectral and chromatographic properties of the *Brassica* anthocyanins

Pigments	λ_{max} in MeOH–HCl (nm) (log ε)			Ratios %		R_f (× 100) in solvent				
	Band I	Band II	Band III	Band II Band III	Band I Band III	1	2	3	4	5
1 Cyanidin-3-sophoroside-5-glucoside	278(4.29);		524(4.57)		52	26	32	14	56	80
2 Cyanidin-3-malonylsophoroside-5-glucoside	278(4.49);		528(4.51)		90	38	43	32	45	77
3a Cyanidin-3-sinapylsophoroside-5-glucoside	280(4.37);	330(4.31);	528(4.57)	54	60	42	44	28	36	75
3b Cyanidin-3-(disinapyl)sophoroside-5-glucoside	282(4.30);	330(4.60);	530(4.58)	105	74	45	53	41	30	66
4a Cyanidin-3-ferulylsophoroside-5-glucoside	282(4.38);	328(4.27);	528(4.57)	50	63	46	49	34	32	74
4b Cyanidin-3-(diferulyl)sophoroside-5-glucoside	282(4.52);	330(4.59);	530(4.54)	103	86	51	57	48	25	66
5a Cyanidin-3-*p*-coumarylsophoroside-5-glucoside	282(4.44);	310(4.35);	526(4.58)	58	71	49	52	39	37	68
5b Cyanidin-3-(di-*p*-coumaryl)-sophoroside-5-glucoside-	282(4.61);	310(4.58);	528(4.57)	102	110	55	64	54	24	63

Solvent key: 1, BAW (4:1:5); 2, BAW (4:1:2); 3, BuOH–2N HCl (1:1); 4, 1% aq.HCl; 5, HOAc–conc. HCl–H_2O (15:3:82). Support Eastman cellulose TLC plates.

with MeOH (0.1% HCl) for 3–4 min during stirring. Approx 95% of the anthocyanins was extracted with only a small amount of chlorophyll present. Extract was filtered and evaporated to *ca* 1 ml. Residue was taken up in 10 ml MeOH (0.01% HCl) and the anthocyanins precipitated Et_2O. The ppt. was filtered, dissolved again in 20 ml MeOH, repptd with Et_2O, and dried. Yield: 0.45 g red powder. A total of 1.2 g crude pigment was isolated in this way. 1.2 g Pigment was dissolved in 50 ml 30% aq. EtOH (2 ml 2N HCl/*l*), adsorbed on a 2 × 35 cm PVP column prepared in H_2O, and the column washed with H_2O (400 ml). The anthocyanins were separated and eluted with 30% aq EtOH (2 ml 2N HCl/*l*). The pigment fractions were evaporated to dryness at 25°, dissolved in a minimal amount of MeOH, precipitated with EtO_2, and dried. Fractions 1 & 2 were rechromatographed on PVP; fractions 3, 4, and 5 were further purified and separated using descending PC in solvent 2. Each fraction gave 2 pigment bands. These were repeatedly purified via PC, eluted from the paper, precipitated with Et_2O, and dried. Yields of pure pigments: *Fr. 1*: 18 mg, *Fr. 2*: 12 mg, *Fr. 3a*: 14 mg; *Fr. 3b*: 18 mg; *Fr. 4a*: 17 mg; *Fr. 4b*: 15 mg; *Fr. 5a*: 14 g and *Fr. 5b*: 16 mg. Alkaline hydrolysis of the pigments and identification of the hydrolysis products was carried out according to [5].

Identification of malonic acid as acylating agent. Et_2O extract from pigment 2 was evaporated to dryness, and the residue silylated [6]. Malonic acid was identified as the silyl derivative with an authentic reference on a Carle AGC-211 gas chromatograph (Column: 6′ length; I.D. 0.085″, 8% OV 101 on Chromosorb W-hp, 100–120 mesh. Column temp: 100°, Carrier gas N_2, 24 ml/min, FID).

Hydrolysis of the pigments and identification of the acyl sugar. This was carried out with 5 mg pigment material on Dowex 50W–X8 as described elsewhere [5].

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ANTHOCYANIN PIGMENTS IN *CALLISTEPHUS CHINENSIS*

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Key Word Index—*Callistephus chinensis*; Compositae; non-acylated and acylated anthocyanins; chemical genetics.

Abstract—Identification of the anthocyanin pigments in the flowers of six genotypes of *Callistephus chinensis* has confirmed that a series of multiple alleles, R, r′ and r are responsible for the production of delphinidin, cyanidin, and pelargonidin derivatives respectively. However, mixtures of anthocyanidin types were present in all genotypes. In the presence of gene M, mainly 3,5-diglycosides were found; in recessive genotypes (mm) there were only 3-monoglucosides. Unstable acylated derivatives of these pigments were also present.

INTRODUCTION

In an extensive paper on the genetics of *Callistephus chinensis*, Wit [1] postulates a series of multiple alleles, R, r′ and r, governing the production of delphinidin, cyanidin, and pelargonidin respectively. He also reported a gene M determining the glycosidic type of the anthocyanins. In the presence of MM and Mm, there are two sugar residues attached to the anthocyanidin; in recessive genotypes (mm) there is only one. Although Wit's results have been questioned on several counts by Beale [2], no further investigations have been carried out. The presence of multiple alleles introduces the concept of a stepwise mutation of genes to produce, ultimately, homologous enzymes with different qualitative effects [3]. For this reason alone, a confirmation of Wit's results is of some importance.