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].

REFERENCES

1. Harborne, J. B. (1964) *Phytochemistry* **3**, 151.
2. Stroh, H. H. and Seidel, H. (1965) *Z. Naturforsch.* **20b**, 39.
3. Tanchev, S. S. and Timberlake, C. F. (1969) *Phytochemistry* **8**, 1825.
4. Birkofer, L., Kaiser, C. Donike, M. and Koch, W. (1965) *Z. Naturforsch.* **20b**, 424.
5. Hrazdina, G. and Franzese, A. J. (1974) *Phytochemistry* **13**, 225.
6. Hrazdina, G. (1970) *J. Agric. Food Chem.* **18**, 243.

Phytochemistry, 1977, Vol. 16, pp. 299–301, Pergamon Press, Printed in England.

ANTHOCYANIN PIGMENTS IN *CALLISTEPHUS CHINENSIS*

GERT FORKMANN

Institut für Biologie II, Lehrstuhl für Genetik, Universität Tübingen, Auf der Morgenstelle 28, BRD

(*Revised received* 7 *August* 1976)

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.

Table 1. Chromatographic and spectral properties of the delphinidin-based pigments of the genotype RRmm

Pigment	R_f^* (× 100) in BAW	BuHCl	1% HCl	HOAc–HCl
I (Dp 3-glucoside)	18	07	02	17
II (acylated deriv)	25	17	03	23
III (acylated deriv)	41	29	06	37

Pigment	spectral properties: λ_{max} in MeOH–HCl (nm)	E_{440}/E_{max} (as %)	E_{310}/E_{max} (as %)
I	276, 535	20	25
II	274, 537	19	23
III	274, 537	20	24

*All R_f's on 0.1 mm cellulose TLC plates (Schleicher and Schüll). On controlled hydrolysis band III gave band II in a few minutes, which then gave band I and delphinidin. Band II gave band I and delphinidin.

RESULTS AND DISCUSSION

Flower pigments of each genotype were separated, purified and analysed by standard procedures [4]. Typical chromatographic and spectral data are shown in Table 1 and the complete results on all genotypes are collected in Table 2. There were some problems in identification and these are considered in respect of the pigments of genotype RRmm. One pigment was readily identified as delphinidin-3-glucoside but the other two components could only be partly characterized (see Table 1). Both gave delphinidin-3-glucoside on alkaline hydrolysis and this and other evidence indicates that they are acylated derivatives. However, the acyl group was not a hydroxycinnamic acid (from UV evidence) and is possibly aliphatic in nature. Similar unstable, incompletely characterized acylated anthocyanins have been reported before from other plants [5–14]. Analyses of the other two mm genotypes gave in the same way cyanidin and pelargonidin 3-glucosides, accompanied in each case by two acyl derivatives.

The pigments of the MM genotype only differed from the above compounds in having an extra sugar at the 5-position. Thus delphinidin, cyanidin and pelargonidin 3,5-diglucosides were present, again each pigment being accompanied by two acyl derivatives (Table 2).

Wit's [1] hypothesis concerning the existence of multiple alleles at the R-locus, doubted by Beale [2], has thus been confirmed. The possibility of two closely linked genes is improbable, because no recombinants were found among 8900 plants so far tested [15]. However, our chromatographic results differ from those of both Wit [1] and Beale [2] in that genotype r'r' contained in addition to the main cyanidin derivatives considerable amounts of pelargonidin and delphinidin glycosides. In genotype RR similarly there are indeed chiefly delphinidin glycosides but also cyanidin and possibly pelargonidin glycosides. Wit [1] found in these genotypes only one or at most two types of aglycones, whereas Beale [2] reported a mixture of delphinidin and pelargonidin derivatives. Thus in our material the degree of oxidation of the anthocyanidin molecule is not completely under genetic control, since the effects of the alleles R, r' and r, only determine which major anthocyanidin is produced. This may be related to Wit's [1] hypothesis that another locus I in the genetic background is responsible for a general increase in the degree of oxidation of the anthocyanidins and that allels R, r' and r simply code for hydroxylases with different substrate specifities.

Plant material. Flowers of six pure lines of *Callistephus chinensis* with the gene combinations RRMM, r'r'MM, rrMM, RRmm, r'r'mm and rrmm were used. The material came initially from Institut für Vererbungs- und Züchtungsforschung der Technischen Universität Berlin. It was further developed in Lehrstuhl für Genetik der Universität Tüblingen so that the above mentioned six pure lines could be investigated in the presence of an isogenic background. The genetic background contains in all cases II and PaPa according to Wit[1].

Isolation and purification. Flowers were collected in September 1975. Only the anthocyanin containing parts of the flowers were used for the extraction of the pigments with 2 × 0.1% MeOH–HCl within 48 hr at 4°. The MeOH–HCl extracts were combined and concentrated. The pigments were redissolved in distilled water, washed with Et_2O (× 3), and with ETOAc (× 3).

Table 2. Anthocyanin pattern of the six genotypes

Pigment/genotype	rrmm	r'r'mm	RRmm	rrMM	r'r'MM	RRMM
Pg -3-glucoside (I)	++	+	−			
Pg -3-glucoside acylated (II)	++++	++	+−			
Pg-3- glucoside acylated (III)	++	+	−			
Cy -3-glucoside (I)	+	++	+		traces	
Cy -3-glucoside acylated (II)	++	+++	++		of	
Cy -3-glucoside acylated (III)	+−	++	+−		3-monoglucosides	
Dp -3-glucoside (I)	−	+	++			
Dp -3-glucoside acylated (II)	−	++	++++			
Dp -3-glucoside acylated (III)	−	+	++			
Pg -3,5-diglucoside (I)				++	+	−
Pg -3,5-diglucoside acylated (II)				++++	++	+−
Pg -3,5-diglucoside acylated (III)		No		++	+	−
Cy -3,5-diglucoside (I)		evidence		+	++	+
Cy -3,5-diglucoside acylated (II)		of		++	+++	++
Cy -3,5-diglucoside acylated (III)		any		?	?	?
Dp -3,5-diglucoside (I)		3,5-diglucosides		−	+	++
Dp -3,5-diglucoside acylated (II)				−	++	+++
Dp-3,5-diglucoside acylated (III)				−	?	?

Key: concentrations: ++++ very high; +++ high; ++ medium, + low; +− traces; − not detectable.

Chromatography. The following solvent systems were used: BAW, *n*-BuOH–HOAc–H_2O (4:1:5 and 6:2:2); BuHCl, *n*-BuOH–2N HCl (1:1); HOAc–HCl, HOAc–conc. HCl–H_2O (3:1:8 and 15:3:82); dil. HOAc (85:15); dil. HCl (97:3); Bu-HCl–W, *n*-BuOH–HCl–H_2O (5:1:2).

Purification of anthocyanins. A concentrated extract was streaked on paper MN 218 (Machery and Nagel) and separated with BAW for 24–36 hr. Bands were further purified by successive chromatography in dil. HOAc and BAW on paper 2043b and 2945b (Schleicher and Schüll), pigments being eluted from the paper with MAW (MeOH–HOAc–H_2O, 70:5:25).

Spectral analysis. Purified pigments in MeOH containing 0.1% HCl were used to give absorptivities of 0.60–0.90 at the visible maximum.

Acid hydrolysis. Total hydrolysis of crude extracts or the purified pigments was in 10% HCl at 100°C for 1 hr. The aglycones were extracted with amyl alcohol and chromatographed, along with appropriate markers, on 0.1 mm cellulose TLC plates (Schleicher and Schüll) using HOAc–HCl. The aqueous hydrolysate was vacuum concentrated and chromatographed, with authentic sugars, with BAW (6:2:2) on 0.5mm cellulose TLC plates. Controlled hydrolysis of the purified pigments was in 10% HCl at 60–70° for at least 1 hr. Aliquots were examined on 0.1mm cellulose TLC plates using HOAc–HCl and Bu–HCl–W.

Alkaline hydrolysis. Alkaline hydrolysis was in 10% KOH under N_2 in the dark at 25° for 1 hr [14]. After acidification, the reaction mixture was evaporated to dryness under vacuum and the solid residue extracted with *n*-PrOH (3 × 1ml). The PrOH extract was evaporated to dryness and the residue redissolved in MeOH. Portions of this solution were chromatographed, with samples of the crude extract and authentic samples from *Matthiola incana*, on paper 2043b (Schleicher and Schüll) using BAW. An additional portion of each MeOH extract was evaporated to dryness, extracted with Et_2O (3 × 1ml) which was evaporated to dryness, and the residue redissolved in MeOH. This solution was spectrally examined and chromatographed along with hydroxycinnamic acid markers on 0.1mm cellulose TLC plates using BAW.

Acknowledgment—These investigations were supported by a grant from the Deutsche Forschungsgemeinschaft.

REFERENCES

1. Wit, F. (1937) *Genetica* **19**, 1.
2. Beale, G. H. (1941) *J. Genetics* **42**, 197.
3. Alston, R. E. (1964) In *Biochemistry of Phenolic Compounds* (Harborne J. B. ed.), p. 171. Academic Press, London.
4. Harborne, J. B. (1967) *Comparative Biochemistry of the Flavonoids*. Academic Press, New York.
5. Timberlake, C. F., Bridle, P. and Tanchev, S. S. (1971) *Phytochemistry* **10**. 165.
6. Hess, D. and Meyer, C. (1962) *Z. Naturforsch* **176**, 853.
7. Harborne, J. B. and Gavazzi, G. (1969) *Phytochemistry* **8**, 999.
8. Lowry, J. B. (1972) *Phytochemistry* **11**, 725.
9. Anderson, D. W., Gueffroy, D. E., Webb, A. D. and Kepner, R. E. (1970) *Phytochemistry* **9**, 1579.
10. Fong, R. A., Webb, A. D. and Kepner, R. E. (1974) *Phytochemistry* **13**, 1001.
11. Bloom, M. and Geissman, T. A. (1973) *Phytochemistry* **12** 2005.
12. Stafford, H. A. (1965) *Plant Physiol.* **40**, 130.
13. Fong, R. A., Kepner, R. E. and Webb, A. D. (1971) *Am. J. Enol. Vit.* **22**, 150.
14. Anderson, D. W., Julian, E. A., Kepner, R. E. and Webb, A. D. (1970) *Phytochemistry*, **9**, 1569.
15. Seyffert, W. private communication (1976).

Phytochemistry, 1977, Vol. 16, pp. 301–302, Pergamon Press, Printed in England.

STRUCTURE OF ANIBA-DIMER-A ISOLATED FROM *ANIBA GARDNERI**

YVONNE P. MASCARENHAS† and OTTO R. GOTTLIEB‡

†Instituto de Física e Química de São Carlos, Universidade de São Paulo, 13560 São Carlos, São Paulo;
‡Instituto de Química, Universidade de São Paulo, c.p. 20780, São Paulo, Brasil

(*Received* 12 *July* 1976)

Key Word Index—*Aniba gardneri*; Lauraceae; aniba-dimer-A; *rel*-(1*R*,6*S*,7*S*,8*S*)-5-methoxy-7-phenyl-8-[6-(4-methoxy-2-pyronyl)]-1-(*E*)-styryl-2-oxabicyclo[4,2,0]octa-4-en-3-one.

Abstract—Aniba-dimer-A from *Aniba gardneri* (Lauraceae) is shown by X-ray crystallography to be *rel*-(1*R*,6*S*,7*S*,8*S*)-5-methoxy-7-phenyl-8-[6-(4-methoxy-2-pyronyl]-1-(*E*)-styryl-2-oxabicyclo[4,2,0]octa-4-en-3-one.

Aniba-dimer-A (**1**), a constituent of *Aniba gardneri* (Meissn.) Mez (Lauraceae), was synthesized by exposure of the co-occurring monomer 5,6-dehydrocavain (**2a**) to sunlight. The structure proposed for aniba-dimer-A (**1**) relied on a consideration of NMR and MS data [2,3]. As already noted with respect to the analogous photodimer of tri-*O*-methylhispidin (**2b**) [4], it is not possible to distinguish between the four possible stereoisomers by consideration of vicinal coupling constants for the cyclobutane protons. The sole configurational detail

* Part 38 in the series 'The Chemistry of Brazilian Lauraceae'. For Part 37 see ref [1]. Sponsored by Conselho Nacional de Desenvolvimento Científico e Tecnológico, Fundação de Amparo à Pesquisa do Estado de São Paulo, Banco Interamericano de Desenvolvimento and Banco Nacional de Desenvolvimento Econômico.