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PIGMENTS OF CENTROSPERMAE—VII.

BETACYANINS FROM *GOMPHRENA GLOBOSA* L.*

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Abstract—Characterization of seven betacyanins from *Gomphrena globosa* inflorescences is reported. Two of these pigments, gomphrenin-I and gomphrenin-II, have been shown to be 6-*O*-β-D-glucopyranosides of betanidin and isobetanidin, respectively. The other five pigments were proved to be hydroxycinnamoyl (*trans* feruloyl, *trans p*-coumaroyl or *cis p*-coumaroyl) derivatives of gomphrenin-I or gomphrenin-II. For gomphrenins-III, -V and -VI, the number and position of the acyl group in the molecule have also been established.

INTRODUCTION

IN A previous paper on the distribution of betacyanins in the Centrospermae,[1] it was reported that the violet inflorescence of *Gomphrena globosa* L. (globe amaranth) contains, besides small amounts of amarantin, isoamarantin and celosianin whose structures have been recently elucidated,[2–4] eight other violet pigments (gomphrenins -I to -VIII). In the present study, the gomphrenins have been investigated in more detail, except gomphrenin-IV which is present in very small amounts and could not be obtained in a sufficiently pure state. The other gomphrenins have been partially or totally characterized. Gomphrenin-I and -II have been shown to be 6-*O*-β-D-glucopyranosides of betanidin and isobetanidin, respectively, the remaining five gomphrenins being acyl derivatives of the first two.

METHODS AND RESULTS

Isolation

Gomphrenins were isolated from aqueous extracts of the inflorescences of *G. globosa* by chromatography on strongly acid exchange resin and subsequent chromatography on polyamide.[2–4] The pigments, which were contaminated with hydroxycinnamic acids, were purified by ether precipitation from methanol. Due to the complexity of the mixture, separation of the components was extremely difficult and gomphrenins were obtained in a rather low yield.

Gomphrenin-I and -II

Gomphrenin-I was previously reported to give on acid hydrolysis the diastereoisomeric aglycones betanidin (I) and isobetanidin (II), and gomphrenin-II only isobetanidin.[1] When

* Part VI, L. MINALE, M. PIATTELLI, S. DE STEFANO and R. A. NICOLAUS, *Phytochem.* **5**, 1037 (1966).

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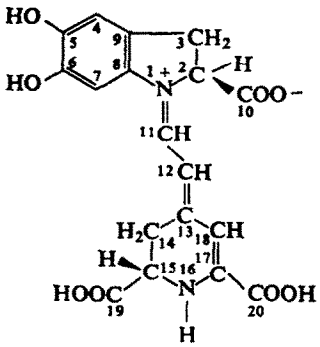
[1] M. PIATTELLI and L. MINALE, *Phytochem.* **3**, 547 (1964).

[2] M. PIATTELLI, L. MINALE and G. PROTA, *Ann. Chim.* **54**, 963 (1964).

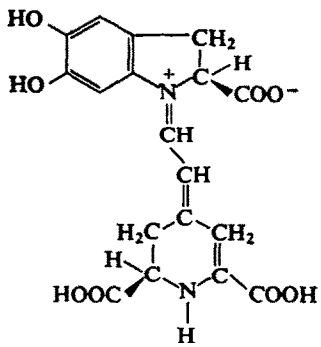
[3] M. PIATTELLI and L. MINALE, *Ann. Chim.* **56**, 1060 (1966).

[4] L. MINALE, M. PIATELLI, S. DE STEFANO and R. A. NICOLAUS, *Phytochem.* **5**, 1037 (1966).

either gomphrenin-I or gomphrenin-II was treated with dilute alkali at room temperature in the absence of oxygen, a 6:4 mixture of gomphrenin-II and gomphrenin-I was obtained. Since, as known, betacyanins in alkaline medium readily undergo partial inversion at the C-15 carbon atom of the aglycone,[5] it was deduced that gomphrenin-I and gomphrenin-II are C-15 diastereoisomers. Furthermore, taking into account the result of the acid hydrolysis, the former pigment is evidently a betanidin and the latter the corresponding isobetanidin derivative.



(I)

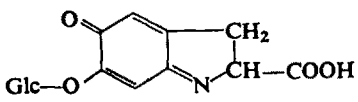


(II)

Additional work on the structure of these two compounds was carried out using a mixture of them, more easily obtainable than the individual pigments. The sugar obtained by acid hydrolysis was chromatographically identified as glucose, and since diazomethane methylation of gomphrenin-I–gomphrenin-II mixture followed by alkali fusion gave 5-methoxy-6-hydroxyindole-2-carboxylic acid, it was inferred that the sugar moiety is linked to the hydroxyl group at position 6 of the aglycone. That gomphrenin-I and -II are monoglucosides was shown by hydrogen peroxide oxidation, which gave glucose.* The pigments mixture was neither hydrolysed by almond emulsin nor attacked by maltase. However, the following evidence suggests that the glucosidic link is β. When the gomphrenin-I–gomphrenin-II mixture was treated with dilute alkali it gave, besides the equilibrium mixture, a compound fluorescing in u.v. light and reacting positively with diazotized sulphanilic acid and Ehrlich reagent which was identified as 5,6-dihydroxyindole-2-carboxylic acid 6-*O*-β-D-glucopyranoside (III).† The identification was based on: (i) β-glucosidase hydrolysis, which gave glucose and 5,6-dihydroxyindole-2-carboxylic acid, and (ii) diazomethane methylation followed by alkali

* In the experimental conditions used for this degradation di- and oligo-saccharides are unaffected by hydrogen peroxide.

† Air oxidation of 2,3-dihydro-5,6-dihydroxyindole-2-carboxylic acid 6-glucoside formed by hydrolysis of gomphrenin-I and -II probably proceeds via the quinonoid intermediate A. In agreement with this hypothesis,

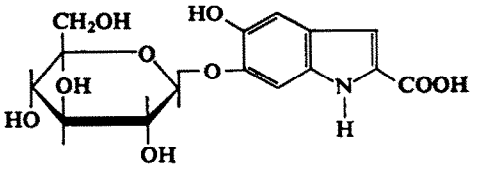


(A)

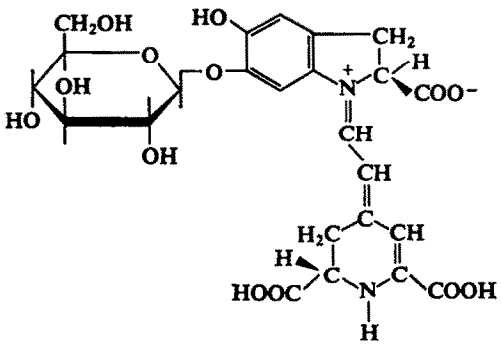
5,6-dihydroxyindole-2-carboxylic acid 5-glucoside is not formed when betanin (5-*O*-β-D-glucopyranoside of betanidin) is treated with alkali.

[5] H. WYLER and A. S. DREIDING, *Helv. Chim. Acta* **42**, 1699 (1959).

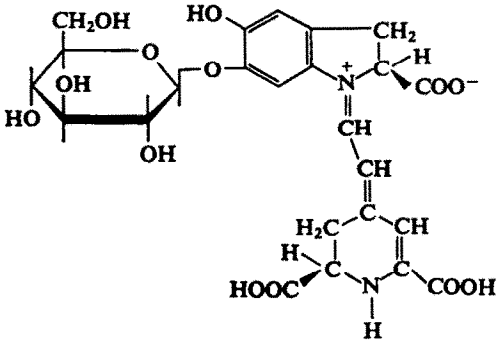
fusion, which yielded 5-methoxy-6-hydroxyindole-2-carboxylic acid. These observations are completely consistent with structures IV and V for gomphrenin-I and -II, respectively. The fact that these pigments are not attacked by almond emulsin may be probably ascribed to steric hindrances to the formation of the enzyme-substrate complex.



(III)



(IV)



(V)

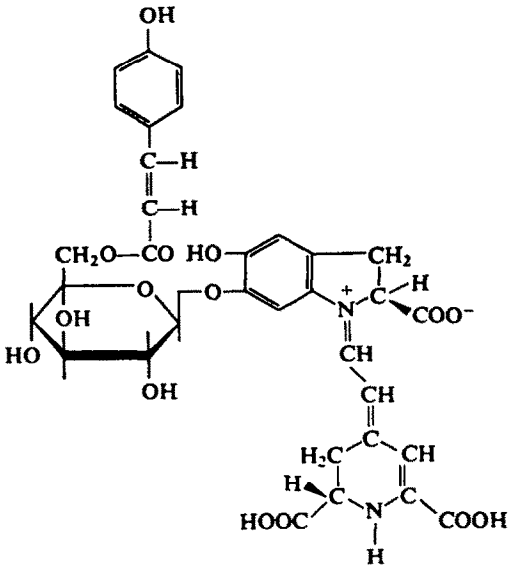
When preliminary investigation on the other gomphrenins showed that all were acyl derivatives of gomphrenin-I or -II, a larger amount of these latter pigments was prepared by alkaline hydrolysis in the absence of oxygen of a crude total mixture of gomphrenins. A crystalline mixture of the two diastereoisomers was obtained ($E^{1\,cm}_{1\%}$ = 920 at 540 nm in water) and its NMR spectrum, measured in CF_3COOH, was consistent with the assigned structures. The spectrum, similar to that of betanin (5-*O*-β-D-glucopyranoside of betanidin[6]) showed the anomeric proton resonance at 5·18δ; the coupling constant (8 c/s) accords with the assigned β-configuration.

Gomphrenin-III

Gomphrenin-III (λ_{max} 280, 542 nm) by treatment with alkali in the absence of oxygen yielded *cis-p*-coumaric acid, identified by paper chromatography, thin-layer chromatography and u.v. spectroscopy, and a mixture of gomphrenin-I and -II, identified by direct comparison of its physical and chemical properties with those of authentic samples. 5,6-Dihydroxyindole-2-carboxylic acid 6-*O*-β-D-glucopyranoside was also obtained. Since diazomethane methylation of gomphrenin-III, followed by alkali fusion of the methylated compound, gave 5-methoxy-6-hydroxyindole-2-carboxylic acid, the phenolic hydroxyl group at position 5 of the aglycone is free in the pigment. Reaction of gomphrenin-III with methyl iodide in dimethylformamide in the presence of silver oxide yielded a permethylated product which was subjected both to acid and alkaline hydrolysis. 2,3,4-Tri-*O*-methyl-D-glucose was

[6] M. PIATTELLI, L. MINALE and G. PROTA, *Ann. Chim.* **54**, 955 (1964).

identified among the products of acid hydrolysis and *p*-methoxycinnamic acid among those of the alkaline hydrolysis.* From this it follows that only one acyl residue is present in the pigment and is linked to the hydroxyl group at position 6 of the glucose unit. The position of attachment of the *cis-p*-coumaroyl group was confirmed by subjecting gomphrenin-III to periodate oxidation followed by borohydride reduction, mild acid hydrolysis and further borohydride reduction, which gave, as expected, glycerol and ethylene glycol. Since gomphrenin-III by acid hydrolysis yielded a mixture of betanidin and isobetanidin, it could not be decided whether it is the betanidin derivative VI or a mixture of VI and its C-15 diastereoisomer.



(VI)

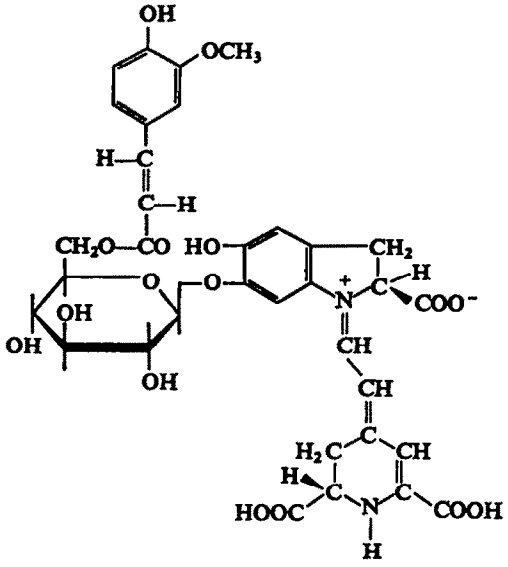
Gomphrenin-V and -VI

Gomphrenin-V (λ_{max} 290, 319, and 549 nm; $E_{319}/E_{549}=0{\cdot}41$) by alkaline hydrolysis gave a mixture of gomphrenin-I and -II, *trans*-ferulic acid and 5,6-dihydroxyindole-2-carboxylic acid 6-*O*-β-D-glucopyranoside. No acyl group is linked to the phenolic hydroxyl, since diazomethane methylation of the pigment followed by alkali fusion yielded 5-methoxy-6-hydroxyindole-2-carboxylic acid. That only one feruloyl group is present in the molecule of gomphrenin-V, and that it is linked to the hydroxyl group at position 6 of the glucose is supported by the following evidence. (i) The acid hydrolysis yielded, besides betanidin, isobetanidin, glucose and *trans*-ferulic acid, another substance isolated by chromatography on polyamide powder and subsequent chromatography on paper (λ_{max}^{EtOH} 325 nm, $\lambda_{max}^{EtOH—NaOH}$ 380 nm), which was identified as *O*-feruloyl-D-glucose, since upon alkaline hydrolysis it gave glucose and *trans*-ferulic acid; (ii) alkaline hydrolysis of the permethylated compound obtained by methylation with methyl iodide and silver oxide in dimethylformamide of gomphrenin-V, yielded 3,4-dimethoxycinnamic acid and no ferulic acid; (iii) acid hydrolysis of the same permethylated compound gave 2,3,4-tri-*O*-methyl-D-

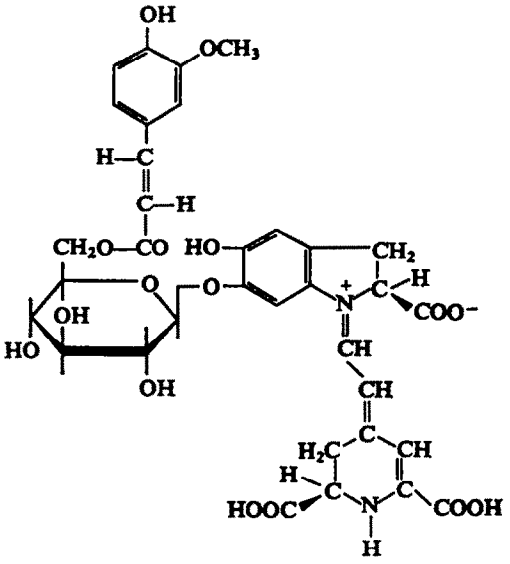
* *p*-Methoxycinnamic acid is destroyed during the acid hydrolysis.

glucose; and (iv) periodate oxidation of gomphrenin-V, followed by borohydride reduction, mild acid hydrolysis and further borohydride reduction yielded glycerol and ethylene glycol.

Gomphrenin-VI (λ_{max} 290, 319, and 549 nm; $E_{319}/E_{549}=0{\cdot}40$) was proved to be C-15 diastereoisomer of gomphrenin-V: in fact it differs from the latter only in yielding on acid hydrolysis isobetanidin and not the mixture of the two aglycones,* the other chemical properties being identical. Therefore structures VII and VIII can be assigned to gomphrenin-V and gomphrenin-VI, respectively.



(VII)



(VIII)

Gomphrenin-VII and -VIII

Gomphrenin-VII (λ_{max} 295, 318, and 549 nm; $E_{318}/E_{549}=0{\cdot}47$) by alkaline hydrolysis yielded a mixture of gomphrenin-I and -II, and *trans*-ferulic acid; alkaline hydrolysis of gomphrenin-VIII (λ_{max} 315 and 549 nm; $E_{315}/E_{549}=0{\cdot}46$) yielded the same deacylated pigments and *trans-p*-coumaric acid. Lack of material prevented an extensive investigation of these compounds.

EXPERIMENTAL

Hydrogen peroxide oxidation, diazomethane methylation followed by alkali fusion, methylation with methyl iodide and subsequent acid or alkaline hydrolysis, and periodate oxidation of pigments were carried out as described previously.[4] Identification of sugars, methylated sugars, *trans*-hydroxycinnamic acids and polyols has also been described.[4]

Ultra-violet spectra were measured on a Beckman DK2 spectrophotometer; absorption maxima of gomphrenins in the visible region found in the present paper, slightly displaced in respect to those reported in a previous work,[1] are more reliable since measurements were carried out on purer compounds.

* In a previous paper[1] gomphrenin-VI was reported to give on acid hydrolysis a mixture of betanidin and isobetanidin; this was probably due to the contamination with gomphrenin-V, complete separation of these two pigments being extremely difficult.

Isolation of Pigments

Violet inflorescences of *Gomphrena globosa* (kg 1·2) were homogenized in a blendor under ice water (8 l.) and extracted for 12 hr at 4°. The aqueous extract was filtered through cheesecloth, and the solid residue re-extracted with ice water (2 l.). The combined extracts at 5°, were adjusted to pH 3 (N HCl) and centrifuged. The supernatant was percolated through a column of Dowex 50W-X2 (H^+ form, 20 × 5 cm) at 5°. The column was washed with 0·1% HCl and eluted with water. The eluant was vacuum concentrated at 30° to about 100 ml and chromatographed in two portions on a 30 × 5 cm powdered polyamide column (5°) with 1 l. of each of 5% citric acid in water, and in 20%, 30%, 40% and finally 50% methanol. After removal of two minor bands (amarantin-isoamarantin and celosianin), four betacyanin fractions were obtained, which emerged from the column after about 1·6, 2·4, 3·5 and 4·6 l., respectively. Each fraction, freed from citric acid by resin treatment, was rechromatographed on a polyamide column (40 × 5 cm). *Fraction* 1, when eluted with 5% citric acid in 20% methanol, yielded two bands which, after removal of citric acid, were vacuum evaporated to dryness giving crude gomphrenin-I (22 mg) and gomphrenin-II (16 mg). *Fraction* 2 (eluent: 5% citric acid in 25% methanol) gave a main violet band (gomphrenin-III, 21 mg) and a minor band, gomphrenin-IV, which was present in too small quantity to be obtained in a pure state and was not further investigated. *Fraction* 3 (5% citric acid in 30% methanol) yielded two bands, gomphrenin-V (57 mg) and gomphrenin-VI (41 mg). From *fraction* 4 (5% citric acid in 35% methanol as the eluent) two bands were obtained, gomphrenin-VII (11 mg) and gomphrenin-VIII (13 mg). All the betacyanins were further purified by ether precipitation from methanol.

Treatment with Alkali of Pigments

Pigment (5–20 mg) was treated at room temperature with 0·2 N NaOH under hydrogen. After 3 hr the solution was acidified with 2 N HCl and the resulting acid solution was continuously extracted with ethyl acetate for 3 hr. From the aqueous solution the deacylated pigment was recovered by absorption on Dowex 50W-X2 (H^+ form), elution with water and evaporation to dryness *in vacuo*. In the residue, gomphrenin-I and -II were identified by (i) co-electrophoresis on paper with authentic samples at pH 2·4 (0·1 M formic acid), 4·5 (0·05 M pyridine formate) and 8·7 (0·2 M borate buffer), (ii) analytical column chromatography according to a described procedure,[1] and (iii) hydrogen peroxide oxidation, which gave glucose. The ethyl acetate extract was chromatographed on paper in *n*-butanol–pyridine–water, 6:4:3 (BPyW). The bands, located by examining the chromatogram in u.v. light in the presence or absence of ammonia vapour, were cut out and eluted with 96% ethanol.

The eluate of the band, R_f 0·23 (5,6-dihydroxyindole-2-carboxylic acid 6-*O*-β-D-glucopyranoside), was further subjected to paper chromatography in *n*-butanol–acetic acid–water, 12:3:5 (BAW). The band, R_f 0·35, was excised and eluted with 96% ethanol. The eluate (λ_{max} 310–315 nm) was taken to dryness and the residue was subjected to (i) hydrolysis with almond emulsin (at 37° in acetate buffer, pH 5, for 2 hr) which gave glucose, identified by standard procedure, and 5,6-dihydroxyindole-2-carboxylic acid, identified by comparison with an authentic sample[7] in paper chromatography (BAW and 0·01 M HCl), thin-layer chromatography on silica gel (benzene–propionic acid–water, 2:2:1 upper phase, BzPrW)

[7] Prepared according to M. PIATTELLI, E. FATTORUSSO, S. MAGNO and R. A. NICOLAUS, *Tetrahedron* **19**, 2061 (1963).

and paper electrophoresis in 0·2 M borate buffer pH 8·7, and (ii) diazomethane methylation and subsequent alkali degradation (refluxing in 25% aq. NaOH for 20 min) which gave 5-methoxy-6-hydroxyindole-2-carboxylic acid, identified by comparison with an authentic sample, prepared according to Piattelli *et al.*,[6] in u.v. spectroscopy and TLC on silica gel (BzPrW as solvent).

Trans-ferulic and *trans-p*-coumaric acids were identified according to a described procedure.[4]

Cis-p-coumaric acid was identified by direct spectral and chromatographic comparison with an authentic specimen, prepared by u.v. irradiation of *trans-p*-coumaric acid according to Neish.[8]

Acid Hydrolysis of Pigments

Pigments were hydrolysed with 22% HCl at 80° for 5 min. The hydrolysate was evaporated to dryness *in vacuo*, and the aglycone(s) (betanidin and/or isobetanidin) were characterized by E_b* values in paper electrophoresis (pH 2·4, 4·5 and 8·7) and spectral determinations both in water and in 0·2 M borate buffer pH 8·7. The remainder of the residue was used for identification of glucose and, in the case of gomphrenin-V and -VI, also for the isolation of *O*-feruloyl-D-glucose. This was accomplished by chromatography on polyamide powder (50% methanol as eluent) and subsequent band chromatography on paper (R_f 0·56 in BAW). The sugar ester was subjected to alkaline hydrolysis (0·2 N NaOH, 3 hr at room temperature) and the hydrolysate was freed from alkali by passing through a column of Amberlite IRC-50 (H^+ form). The eluate was extracted with ether. Glucose and ferulic acid were identified in the aqueous solution and in the ether extract, respectively.

* E_b equals migration on paper electrophoresis relative to betanin.

[8] A. C. NEISH, *Phytochem.* **1**, 1 (1961).