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PIGMENTS OF CENTROSPERMAE—VI.
ACYLATED BETACYANINS*

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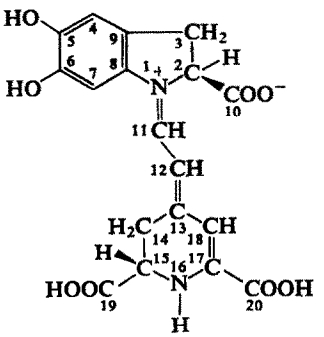
Abstract—Characterization of eight betacyanins from three species plant (*Phyllocactus hybridus, Celosia cristata, Iresine herbstii*) is described. All these pigments have been shown to be acyl derivatives of betanin (III), amarantin (IV), or their corresponding C-15 diastereoisomers (isobetanin and isoamarantin). Both aliphatic (malonic acid and 3-hydroxy-3-methylglutaric acid) and hydroxycinnamic (ferulic, *p*-coumaric, sinapic and caffeic acids) acids are present in the pigments as acyl residues. In seven of the compounds the acyl groups are linked to the sugar residue of the molecule, in the other evidence was obtained that the acid is directly linked to the aglycone moiety. For most of them, the number and position of the acyl group(s) in the molecule has also been established.

INTRODUCTION

IN RECENT YEARS, several betacyanins have been isolated from plants belonging to the order Centrospermae. Preliminary investigation has shown them to be derivatives of betanidin or its C-15 diastereoisomer, isobetanidin;[1] these two aglycones have been shown to have the structures I and II,[2,3] respectively. Until now, the structures of a few betacyanins, namely betanin (5-*O*-*β*-D-glucopyranoside of betanidin, III),[4,5] isobetanin (5-*O*-*β*-D-glucopyranoside of isobetanidin),[4,5] amarantin, i.e. betanidin 5-*O*-[2-*O*-(*β*-D-glucopyranosyluronic acid)-*β*-D-glucopyranoside] (IV)[6,7] and isoamarantin, i.e. isobetanidin 5-*O*-[2-*O*-*β*-D-glucopyranosyluronic acid)-*β*-D-glucopyranoside][6,7] have been elucidated.



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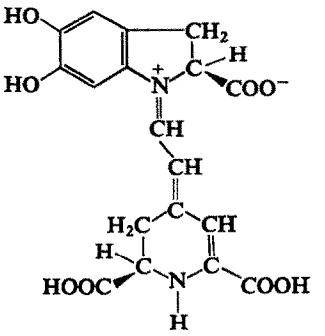

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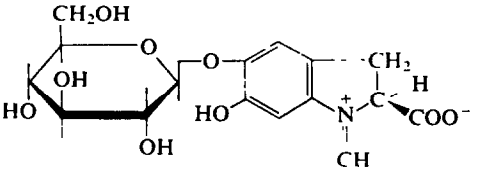
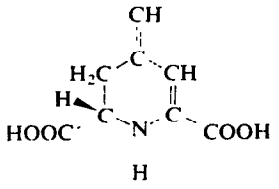
* Part V, M. PIATTELLI, L. MINALE and R. A. NICOLAUS, *Phytochem.* **4**, 817 (1965).

[1] M. PIATTELLI and L. MINALE, *Phytochem.* **3**, 547 (1964).
[2] H. WYLER, T. J. MABRY and A. S. DREIDING, *Helv. Chim. Acta* **46**, 1745 (1963).
[3] M. E. WILCOX, H. WYLER and A. S. DREIDING, *Helv. Chim. Acta* **48**, 1134 (1965).
[4] M. PIATTELLI, L. MINALE and G. PROTA, *Ann. Chim.* **54**, 955 (1965).
[5] M. E. WILCOX, H. WYLER, T. J. MABRY and A. S. DREIDING, *Helv. Chim. Acta* **48**, 252 (1965).
[6] M. PIATTELLI, L. MINALE and G. PROTA, *Ann. Chim.* **54**, 963 (1965).
[7] M. PIATTELLI and L. MINALE (In press).





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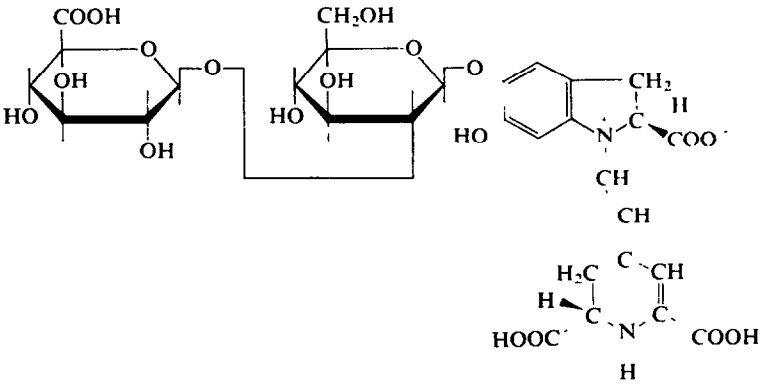


(IV)

The present paper deals with the characterization of eight further betacyanins. The occurrence of six of these pigments has already been reported.[1] All these pigments have been shown to be acylated betacyanins, and the identity and position of attachment of the sugar residue to the aglycone and the identity of acyl substituents have been established in each case. For most of them, the number and position of the acyl group(s) in the molecule have been also determined.

METHODS AND RESULTS

Isolation

The acylated betacyanins were isolated by column chromatography on strongly acid exchange resin and subsequent chromatography on polyamide powder, similar to the procedures described for betanin and amarantin.[6,8]

Betanidin and Isobetanidin Derivatives

It has been previously shown that two betacyanins differing only in the configuration at the C-15 carbon atom can be reversibly transformed under suitable experimental conditions;[1]

[8] M. PIATTELLI and L. MINALE, *Phytochem.* 3, 307 (1964).

it was also observed that betanidin derivatives give, upon acid hydrolysis, a mixture of betanidin and isobetanidin, whereas isobetanidin derivatives give only the latter aglycone.[8] On the basis of these two facts, it is possible to establish whether two betacyanins of unknown structure are C-15 epimers and, if this is the case, which of them is a derivative of betanidin and which of isobetanidin.

Deacylation of Pigments

Treatment of the pigments with alkali, in the absence of oxygen, resulted in the removal of the acyl group(s). After acidification of the reaction mixture, the organic acids were extracted into ethyl acetate and their identity established by direct comparison with known compounds. The aqueous layer was examined for the deacylated pigments and in every case a mixture of two diastereoisomers was obtained, i.e. betanin and isobetanin or amarantin and isoamarantin. This is accounted for by the facile partial inversion at the C-15 carbon atom which occurs in alkaline solution.[9] The deacylated pigments were identified by comparison of their chromatographic and electrophoretic properties with those of reference samples. Since betanin and amarantin isomers, differing in the position of attachment of the sugar residue to the aglycone and/or (in the case of amarantin) in the bond between the sugar units, might have conceivably the same chromatographic and electrophoretic properties of betanin and amarantin, the identification of the deacylated pigments was substantiated by enzymic and chemical degradation.

Number and Position of Acyl Groups

Physical methods (determination of neutral equivalent of pigments, u.v. and NMR spectroscopy) were used in order to determine the molar ratio between the acid(s) and the deacylated pigment.

No pigment proved to have an acyl group linked to the phenolic hydroxyl group at position 6 of the aglycone, since diazomethane methylation, followed by degradation with alkali, always gave 5-hydroxy-6-methoxyindole-2-carboxylic acid. In fact, for only one of the isolated pigments (iresinin-III), was evidence obtained that at least one acid is directly bound to the aglycone moiety. In all the other cases, it was shown that the acyl residues are bound to the sugar moiety by an ester link.

In order to determine the exact position of the acyl group(s) in the molecule, pigments available in sufficient amount were subjected to periodate oxidation. Very mild experimental conditions were used to minimize the possibility of acyl migration. The results so obtained were confirmed by methylation of the pigments with methyl iodide and identification of the products obtained by acid hydrolysis of the permethylated compounds.

Pigments from Phyllocactus hybridus *Hort.*

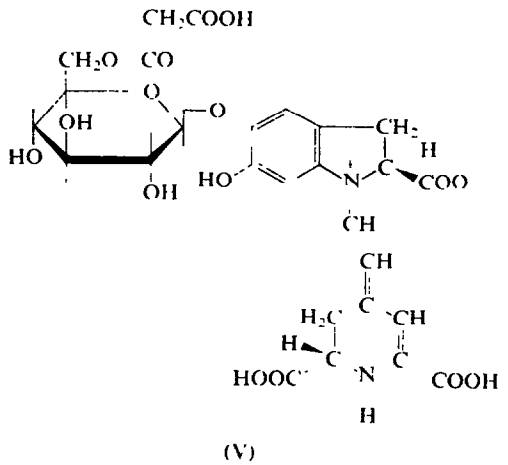
The occurrence of four betacyanins (betanin, isobetanin, phyllocactin and isophyllocactin) in the flowers of *Phyllocactus hybridus* (Cactaceae) has already been described.[1,8] The structures of the latter two pigments were not determined, but they were shown to be C-15 diastereoisomers. Since phyllocactin gave a mixture of betanidin and isobetanidin after acid hydrolysis, whereas isophyllocactin yielded only isobetanidin, it was deduced that phyllocactin is a betanidin, and isophyllocactin an isobetanidin, derivative. Therefore, their further characterization did not require their separation and accordingly the isolation procedure

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

was modified. Shorter and more highly loaded polyamide columns were used, giving better yields on account of the reduced time required for the isolation.

Upon treatment with alkali in the absence of oxygen, the phyllocactin–isophyllocactin mixture (λ_{max} 538 mμ, E^{1cm}_{1} 740 in water; neutral equivalent 221, calc. for $C_{24}H_{25}O_{10}N_2(COOH)_3$ 212) yielded malonic acid, identified by paper chromatography, thin-layer chromatography and high voltage electrophoresis, and gas–liquid chromatography of its methyl ester. The deacylated pigment was unequivocally identified as a mixture of betanin and isobetanin by (i) paper electrophoresis, (ii) analytical column chromatography on polyamide, (iii) treatment with emulsin which gave glucose and a mixture of betanidin and isobetanidin without formation of intermediates (monoglucosides), and (iv) diazomethane methylation and subsequent alkali fusion[4] which gave 5-hydroxy-6-methoxyindole-2-carboxylic acid (5-glucosides). From the neutral equivalent of the pigment only one malonyl residue is present in the molecule and this is not linked to the phenolic hydroxyl group at position 6 of the aglycone as shown by diazomethane methylation and subsequent alkali fusion which gave 5-hydroxy-6-methoxyindole-2-carboxylic acid. The fact that emulsin does not hydrolyse the intact compound suggests that the acyl group is bound to the glucose. In order to obtain more direct evidence, the phyllocactin–isophyllocactin mixture was subjected to mild acid hydrolysis but no *O*-malonyl-D-glucose could be obtained, the lability of this sugar ester in acidic solution accounts for this negative result. We resorted therefore to the oxidation with hydrogen peroxide* which gave, besides glucose and malonic acid, an acidic substance (in paper electrophoresis it migrates as an anion) which appeared to be an *O*-malonyl-D-glucose, since on alkaline hydrolysis it gave glucose and malonic acid.

In order to ascertain the position of malonyl group in the glucose residue, phyllocactin–isophyllocactin was subjected to: (*a*) periodate oxidation followed by borohydride reduction, mild acid hydrolysis and borohydride reduction, which gave glycerol and ethylene glycol; and (*b*) methylation with methyl iodide and silver oxide in dimethylformamide and subsequent acid hydrolysis, which gave 2,3,4-tri-*O*-methyl-D-glucose. These findings are in agreement with the location of the *O*-malonyl group at C-6 of the glucose moiety and the structures of phyllocactin and isophyllocactin are thus V and VI, respectively.



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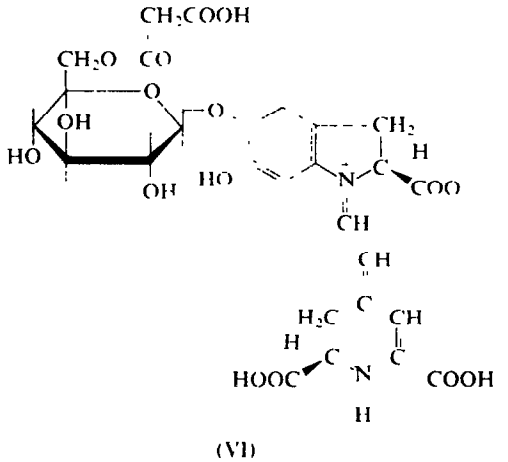


(VI)

* This degradation had been used previously the obtaining sophorobiuronic acid [i.e. 2-*O*-(β-D-glucopyranosyluronic acid)-β-D-glucopyranose] from amarantin.[5]

It should be noted that the experiments to determine the exact position of the acyl group were performed under very mild conditions, so that migration of acyl group is unlikely. However, it cannot be excluded that such a migration might have taken place during the purification process. In fact, a very large number of acyl group migration have been observed in the field of carbohydrate chemistry.[10] If this were true, phyllocactin and isophyllocactin may be artifacts.

Pigments from Celosia cristata *L.*

Among the garden varieties of *Celosia cristata* (Amarantaceae) there is a large colour variation from yellow to various shades of red and violet. Red inflorescences contain amarantin and isomarantin, and the violet ones two further pigments, viz. celosianin and isocelosianin.[1] In the present work violet inflorescences of an unidentified cultivar were used. Since it was already known that celosianin and isocelosianin are C-15 diastereoisomers, they were not separated. A crystalline mixture of the two pigments was obtained, λ_{max} 545 mμ ($E^{1cm}_{1\%}$ 465 in water), 319 mμ ($E^{1cm}_{1\%}$ 242), 300 mμ ($E^{1cm}_{1\%}$ 250); neutral equivalent 356, calc. for $C_{46}H_{45}O_{18}N_2(COOH)_3$ 349·6. Alkaline hydrolysis of the celosianin–isocelosianin mixture gave a deacylated product which was identified as a mixture of amarantin and isoamarantin by (i) paper electrophoresis, (ii) analytical column chromatography on polyamide, (iii) treatment with β-glucuronidase, which gave glucuronic acid and a mixture of betanin and isobetanin, and (iv) methylation with methyl iodide and silver oxide in dimethylformamide followed by acid hydrolysis which gave 3,4,6-tri-*O*-methyl-D-glucose and 2,3,4-tri-*O*-methyl-D-glucuronic acid, thus establishing the exact nature of the carbohydrate unit. The strong absorption in the 300–320 mμ region of the u.v. spectrum of celosianin–isocelosianin, which is not observed in the spectrum of amarantin, pointed to the probable presence of aromatic acyl groups. Actually, the acid fraction obtained by deacylation, when purified by means of band chromatography, gave two acids whose spectral properties showed that they are hydroxycinnamic acids. By comparison with known compounds, they were identified as *trans-p*-coumaric and *trans*-ferulic acids. From the value of the neutral equivalent of celosianin–isocelosianin, it was deduced that 1 mole of amarantin–isoamarantin, 1 mole of ferulic acid and 1 mole of *p*-coumaric acid are present in this pigment. This was further substantiated by consideration of the overall proton integration of its NMR spectrum, which showed the presence of fourteen protons more than those present in the amarantin spectrum, eleven of them in the range 8·2–6·2 δ (aromatic and olefinic protons) and three at 4.02 δ (—OCH_3). Overlapping precluded more specific assignments of bands. Since celosianin–isocelosianin is resistant to β-glucuronidase hydrolysis, at least one acyl group is linked to the glucuronic acid residue. Further information on the position of the acyl groups in the molecule was obtained by acid hydrolysis, which gave, besides betanidin, isobetanidin, glucose, glucuronic acid, glucuronolactone, sophorobiuronic acid [i.e. 2-*O*-(β-D-glucopyranosyluronic acid)-D-glucopyranose], *p*-coumaric and ferulic acids, six compounds, located on the chromatograms by fluorescence in u.v. light on fuming with ammonia vapour. These compounds were isolated by band chromatography. Their chromatographic and electrophoretic properties, and the products obtained by alkaline hydrolysis are reported in Table 1. Identification of a *p*-coumaroylglucose and a feruloylglucuronolactone among the acid hydrolysis products shows that celosianin is a *p*-coumaroyl–feruloylamarantin and isocelosianin a *p*-coumaroyl–feruloylisoamarantin, in which *p*-coumaric acid is bound to the

[10] J. M. SUGIHARA, *Advan. Carbohydrate Chem.* **8**, 1 (1953).

glucose unit and ferulic acid is bound to the glucuronic acid unit. When celosianin–isocelosianin was subjected to periodate oxidation, followed by borohydride reduction and acid hydrolysis, glycerol, glucuronic acid, glucuronolactone and glyceric acid were obtained. Formation of glycerol is in agreement with the location of the *O-p*-coumaroyl group at C-6 of glucose residue. The fact that from the glucuronic acid moiety both glucuronic acid and glyceric acid are formed, may be rationalized on the assumption that: (*a*) the pigment subjected to the degradation was a mixture of two isomeric compounds differing in the position of attachment of the feruloyl group to the glucuronic acid residue (2 or 3); or (*b*) a migration of acyl group between the C-2 and C-3 hydroxyl groups of glucuronic acid residue had occurred during the oxidation. The latter hypothesis is not very likely, since the experimental conditions used for the periodate oxidation were very mild. Should celosianin be actually a mixture of two isomeric compounds with identical electrophoretic and chromatographic properties, the question whether both isomers are present in the living tissues or only one is synthesized in the plant (the other being formed by a migration of the acyl group during the isolation) at the present cannot be determined.

Pigments from Iresine herbstii *Hook*

Leaves of *Iresine herbstii* had earlier been found to contain amarantin and two other pigments, iresinin-I and -II.[1] In the present work, the betacyanin components of *Iresine herbstii* have been reinvestigated in detail and two minor pigments (iresinin-III and -IV) were also isolated.

(*a*) *Iresinin-I and -II.* Iresinin-I was obtained as violet crystals, λ_{max} 537 mμ ($E^{1\%}_{1cm}$ 690 in water); it is optically active $[\alpha]^{20}_{675}+160\pm10°$; neutral equivalent 224, calc. for $C_{32}H_{38}O_{15}N_2$ $(COOH)_4$ 217·7. Iresinin-II was not obtained in crystalline form. That iresinin-I and -II are C-15 diastereoisomers was deduced from their reversible conversion and since iresinin-I upon acid hydrolysis gave a mixture of betanidin and isobetanidin, and iresinin-II yielded only isobetanidin, the former is a betanidin derivative and the latter is an isobetanidin derivative (the name iresinin-II should be accordingly changed to isoiresinin-I). The NMR spectrum of iresinin-I (in trifluoroacetic acid) differs from that of amarantin in the presence of two sharp singlets at 3·00 δ (2 $-CH_2CO-$) and at 1·62 δ ($CH_3-\overset{|}{\underset{|}{C}}-$) (Fig. 1); by alkaline hydrolysis, the pigment gave a mixture of amarantin and isomarantin, identified as described for celosianin, and an optically inactive acid, m.p. 108°. By reaction with diazomethane of this acid a methyl ester was obtained, whose i.r. spectrum (CCl_4) showed a hydroxyl band at 3450 cm^{-1}; from the NMR spectrum (CCl_4), which showed three singlets at 3·65 δ integrating for seven protons (2 $-OCH_3$ and 1 $-OH$), at 2·58 δ (2 $-CH_2CO-$) and at 1·28 δ ($CH_3-\overset{|}{\underset{|}{C}}-$), it was deduced that the acid obtained from iresinin-I is 3-hydroxy-3-methyl-glutaric acid (HMG). The identification was confirmed by direct comparison with an authentic sample. Upon β-glucuronidase treatment iresinin-I yielded glucuronic acid and a red–violet pigment, desglucuronosyliresinin-I, (λ_{max} 537 mμ; E_b*'s: pH 2·4 = 0·96, pH 4·5 = 1·00, pH 8·7 = 1·23), not hydrolysed by emulsin. This pigment on acid hydrolysis gave betanidin, isobetanidin, glucose, HMG and an acidic compound which, when hydrolysed

* E_b equals migration on paper electrophoresis relative to betanin.

TABLE 1. PROPERTIES OF SUGAR ESTERS OBTAINED BY ACID HYDROLYSIS OF CELOSIANIN–ISOCELOSIANIN

Compound	R_f values in BAW	Migration in paper electrophoresis (pH 6·8)*	Colour in u.v.		Products of alkaline hydrolysis†	Identified as
			untreated	NH_3 vapour		
A1	0·25	+	Blue	Green	SA + FA	Feruloylsophorobiuronic acid
A2	0·30	+	Colourless	Blue	SA + *p*-CA	*p*-Coumaroylsophorobiuronic acid
A3	0·53	−	Blue	Green	SA + FA	Feruloylsophorobiuronolactone
A4	0·60	−	Colourless	Blue	Gl + *p*-CA	*p*-Coumaroylglucose
B1	0·66	−	Blue	Green	GlA + FA	Feruloylglucuronolactone
B2	0·75	−	Colourless	Blue	SA + *p*-CA	*p*-coumaroylsophorobiuronolactone

* + = migrates as an anion; − = does not migrate.

† Key: SA = sophorobiuronic acid, Gl = glucose, GlA = glucuronic acid, FA = ferulic acid, *p*-CA = *p*-coumaric acid.

with alkali, yielded glucose and HMG. Hence, HMG is bound to the glucose residue by an ester link. Since desglucuronosyliresinin-I, when subjected to periodate oxidation followed by borohydride reduction, acid hydrolysis and borohydride reduction, gave glycerol and ethylene glycol, HMG is linked to the hydroxyl group at position 6 of the glucose moiety and iresinin-I is betanidin 5-*O*-[2-*O*-(β-D-glucopyranosyluronic acid)-6-*O*-(3-hydroxy-3-methylglutaryl)-β-D-glucopyranoside] (VII). In agreement with the structure proposed for iresinin-I, desglucuronosyliresinin-I by methylation with methyl iodide and silver oxide in dimethylformamide, followed by acid hydrolysis of the permethylated compound, gave 2,3,4-tri-*O*-methyl-D-glucose. In this case, as for phyllocactin and celosianin, it cannot be excluded that an acyl migration had not occurred during the isolation.

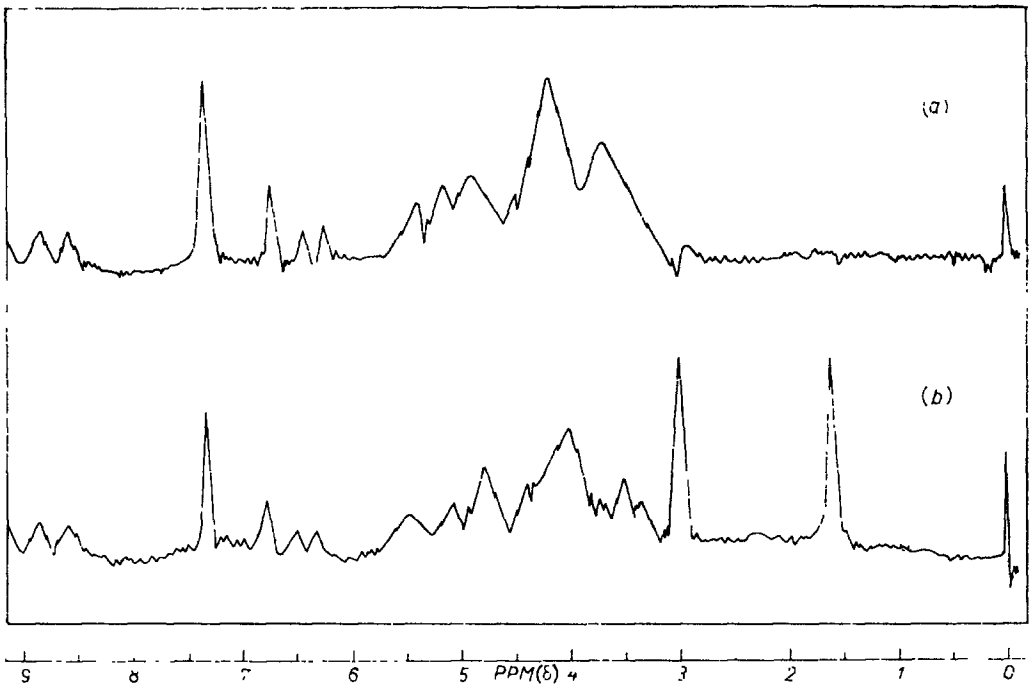
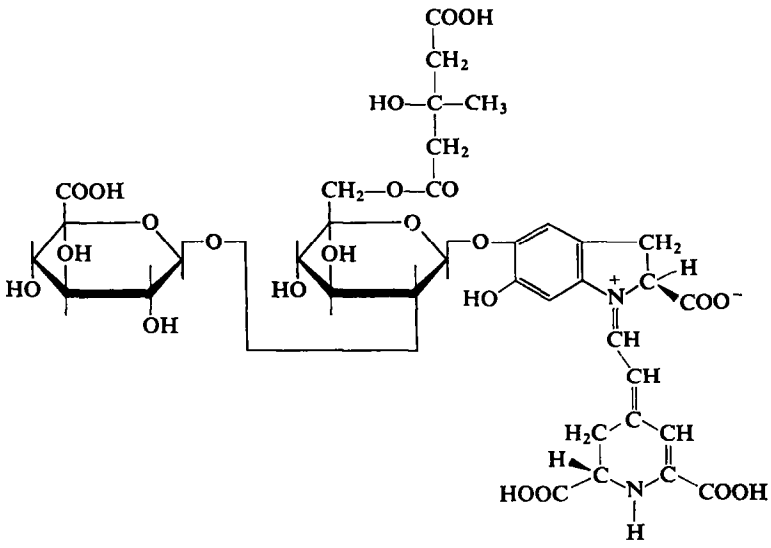


FIG. 1. NMR SPECTRA IN TRIFLUOROACETIC ACID OF (a) AMARANTIN AND (b) IRESININ-I.

Isoiresinin-I (formerly iresinin-II) is evidently isobetanidin 5-*O*-[2-*O*-(β-D-glucopyranosyluronic acid)-6-*O*-(3-hydroxy-3-methylglutaryl)-β-D-glucopyranoside]; in agreement with this structure, it gave upon alkaline hydrolysis the same degradation products as iresinin-I. Desglucuronosylisoiresinin-I, obtained from the pigment by β-glucuronidase treatment, had electrophoretic properties identical to those of desglucuronosyliresinin-I. When hydrolysed with acids, it gave glucose, HMG, *O*-(3-hydroxy-3-methylglutaryl)-D-glucose and isobetanidin.

(*b*) *Iresinin-III.* Iresinin-III (λ_{max} 537 mμ, 298 mμ; E_b's: pH 2·4 = 0·48, pH 4·5 = 0·74, pH 8·7 = 1·11) could not be obtained in crystalline form. By alkaline hydrolysis it yielded amarantin and isoamarantin, identified as above, and *trans*-*p*-coumaric, *trans*-ferulic, *trans*-sinapic and *trans*-caffeic acids, identified by chromatography and u.v. spectroscopy. β-Glucuronidase hydrolysed iresinin-III, giving glucuronic acid and a red violet pigment,



(VII)

desglucuronosyliresinin-III, (E_b's: pH 4·5=0·65, pH 8·7=0·89), thus showing that no hydroxycinnamic acid is linked to the glucuronic acid moiety. Iresinin-III, when subjected to acid hydrolysis, gave neither betanidin nor isobetanidin, but a violet pigment (λ_{max} 544 mμ; E_b's: pH 2·4=0·05, pH 4·5=0·15, pH 8·7=0·55), isolated by paper electrophoresis. The u.v. absorption maximum of this pigment in borate buffer, pH 8·7, showed a bathochromic shift of about 12 mμ. An analogous shift is observed for betanidin but not for its glucosides (betanin, amarantin) and is evidently due to the formation of a complex between *o*-phenolic hydroxyl groups and borate ions. It is therefore likely that in this pigment a carboxyl group of the aglycone (betanidin or isobetanidin) is bound by an ester link to a hydroxycinnamic acid. Iresinin-III is thus the first acylated betacyanin in which the acid is directly linked to the aglycone.

(*c*) *Iresinin-IV.* Iresinin-IV (λ_{max} 545 mμ, 321 mμ, 300 mμ; E_b's: pH 2·4=1·11, pH 4·5=1·16, pH 8·7=1·40) was also not obtained in the crystalline state. Upon alkaline hydrolysis it gave amarantin, isoamarantin and *trans*-ferulic and *trans*-sinapic acids. Since sinapic acid and *p*-coumaric acid have very similar molar absorpivity in the 312–320 mμ region, from the ratio E_{320}/E_{545}=0·54, which is very similar to that of celosianin (*p*-coumaroyl-feruloyl-amarantin), it was inferred that, in iresinin-IV, amarantin–isoamarantin, ferulic acid and sinapic acid are in the molar ratio 1 : 1 : 1. Iresinin-IV was subjected to methylation with methyl iodide and silver oxide in dimethylformamide and the permethylated product was hydrolysed with cold, dilute alkali: paper chromatography of the degradation products showed the presence of 3,4-dimethoxycinnamic acid and 3,4,5-trimethoxycinnamic acid and no ferulic or sinapic acids. From this it follows that ferulic and sinapic acids are bound to the molecule by their carboxyl group. Since the phenolic hydroxyl group at position 6 of the aglycone is free, as shown by diazomethane methylation of iresinin-IV followed by alkali fusion, which gave 5-hydroxy-6-methoxyindole-2-carboxylic acid, both hydroxycinnamic acids are linked to the sugar moiety and at least one of the acids is bound to the glucuronic acid residue, since iresinin-IV is unaffected by β-glucuronidase. It must be also observed that the aglycone obtained by acid hydrolysis of the pigment was identified as a mixture of

betanidin and isobetanidin, and therefore it could not be established whether iresinin-IV is a single pigment based on betanidin or a mixture of two C-15 diastereoisomeric compounds with identical electrophoretic and chromatographic properties.

A more complete characterization of iresinin-III and -IV awaits the isolation of larger amounts of these compounds.

CONCLUSION

The results described in the present paper suggest that acylation is rather common in the field of betacyanins, in contrast with what is observed for the anthocyanins. In the case of the anthocyanins, acylation is almost unusual and is taxonomically restricted to a small number of families (such as Labiateae, Cruciferae, Vitaceae and Solanaceae); furthermore, only three hydroxycinnamic acids (*p*-coumaric, ferulic and caffeic acids) have been shown to be present in these pigments.[11] In the case of betacyanins, on the contrary, besides hydroxycinnamic acids, aliphatic acids are also present, and in the same plant (e.g. *Iresine herbstii*) pigments acylated with both types of acids can co-occur. It should be noted that in the acylated anthocyanins the acyl group is always linked to the sugar moiety; this is also true for seven out of the eight acylated betacyanins characterized so far: however, in the case of iresinin-III evidence was obtained that an acid is directly linked to the aglycone moiety.

EXPERIMENTAL

Plant Materials

Flowers of *Phyllocactus hybridus* were collected in the Naples area. Violet inflorescences of *Celosia cristata* (cockscomb) of an unidentified garden variety were used for the isolation of celosianin–isocelosianin. *Iresine herbstii* was grown from seeds in soil in the glasshouse.

Authentic Pigments

Betanin and isobetanin were isolated from *Beta vulgaris* roots.[8] Amarantin and isoamarantin were isolated from leaves of *Amarantus tricolor* according to a previously described procedure.[5] By acid hydrolysis of betanin and subsequent chromatography on polyamide, using 5% aq. citric acid as the eluent, samples of betanidin and isobetanidin were obtained.

Paper Chromatography (PC)

Paper chromatography was carried out by the descending technique on Whatman No. 1 paper.

(*a*) *Solvents for sugars.* BAW, *n*-butanol–acetic acid–water (12:3:5); BPyW, *n*-butanol–pyridine–water (6:4:3); PEaW, *n*-propanol–ethyl acetate–water (7:1:2); EaPyW, ethyl acetate–pyridine–water (8:2:1).

(*b*) *Solvents for methylated sugars.* BEW, *n*-butanol–ethanol–water (5:1:4, upper phase).

(*c*) *Solvents for polyols.* BAW; BPyW; EaPyW.

(*d*) *Solvents for hydroxycinnamic acids.* BAW; BPyW; EAW1, ethanol–33% ammonia–water (20:1:4); BzAW1, benzene–acetic acid–water (125:72:3); IAW, *iso*propanol–33% ammonia–water (20:1:2); TAW, toluene–acetic acid–water (5:3:5, upper phase); 3% NaCl in 0·1 N HCl.

(*e*) *Solvents for aliphatic acids.* BAW; BPyW; PA, *n*-propanol–33% ammonia (3:2).

(*f*) *Detection of constituents.* Sugars and sugar derivatives were detected with aniline

[11] J. B. HARBORNE, *Phytochem.* 3, 151 (1964).

phosphate. After spraying, papers were heated for 10 min at 105°. 3,4,6-tri-*O*-Methyl-D-glucose was also detected with lead tetracetate (1% solution in benzene). The hydroxycinnamoyl sugar esters were located by fluorescence in u.v. light in the presence or not of ammonia vapour. Aliphatic acids were detected with bromophenol-blue indicator; for phenolic acids, chromatograms were examined in u.v. light in the presence or absence of ammonia vapour and by spraying with diazotized 4-nitro-*o*-anisidine (stabilized; Echtrotsalz B, Fluka AG, Buchs, Switzerland) followed by 2 N NaOH (*p*-coumaric acid: blue; ferulic acid: light blue; sinapic acid: green; caffeic acid: dark yellow). 3,4-Dimethoxycinnamic and 3,4,5-trimethoxycinnamic acids were detected by fluorescence in u.v. light and by spraying the chromatograms with $KMnO_4$. Polyols were detected with periodate reagent.[12]

Thin-layer Chromatography (TLC)

(*a*) *Methylated sugars.* Thin-layer chromatography on silica-gel (Kieselgel G, Merk) was carried out by using the following solvent system: DM, dichloromethane–methanol (9:1).

(*b*) *Aliphatic acids.* Chromatoplates of silica-gel and the following solvents were used: BzAM, benzene–acetic acid–methanol (45:8:4); EAW2, ethanol–25% ammonia–water (25:4:3).

(*c*) *Hydroxycinnamic acids.* Plates of silica-gel and the following solvent system were used: BzAW2, benzene–acetic acid–water (2:1:1, upper phase). R_f values are as follows: caffeic acid, 0·06; *p*-coumaric acid, 0·24; sinapic acid, 0·31; ferulic acid, 0·43; 3,4,5-trimethoxycinnamic acid, 0·51; 3,4-dimethoxycinnamic acid, 0·57; *p*-methoxycinnamic acid, 0·60. Thin-layer chromatography on polyamide powder was carried out by using a mixture of methanol–water (70:30) as the eluent.

(*d*) *Indole derivatives.* Plates of silica-gel and the following solvent system were used: BzPrW, benzene–propionic acid–water (2:2:1, upper phase); 5-hydroxy-6-methoxyindole-2-carboxylic acid R_f 0·65, 5-methoxy-6-hydroxyindole-2-carboxylic acid R_f 0·74.

(*e*) *Detection of constituents.* For detection of methylated sugars and acids the spray reagents described under paper chromatography were used. Indole derivatives were detected by u.v. light, Ehrlich reagent and diazotized 4-nitro-*o*-anisidine oversprayed with 2 N NaOH.

Analytical Chromatography on Polyamide Column

A previously described procedure was used[1]: polyamide column (40 × 0·9 cm); developing solvent: increasing concentrations of methanol in aqueous citric acid.

Gas-liquid Chromatography (GLC)

A gas chromatography apparatus (Fractovap C. Erba, Milano, model PAID/f) equipped with a flame ionization detector was used. All measurements were carried out with a glass capillary column (length: 60 m; liquid-phase: diethylene glycol–succinic acid polyester). Nitrogen (1 ml/1 min) was used as carrier gas; split ratio 1:100.

Paper Electrophoresis

Electrophoretograms were run on Whatman No. 1 paper for about 1 hr at 16 V/cm in a horizontal apparatus. The following electrolytes were used: formic acid 0·1 M (pH 2·4);

[12] I. SMITH, *Chromatographic and Electrophoretic Techniques* (Edited by I. SMITH), Vol. 1, p. 252. William Heinemann-Medical Books and Interscience, New York (1960).

pyridine formate 0·05 M (pH 4·5); phosphate buffer 0·05 M, pH 6·8; phosphate buffer 0·05 M, pH 8; borate buffer 0·2 M, pH 8·7.

Tentative identification of degradation products were always substantiated by mixed chromatography and electrophoresis with authentic samples.

Spectra

Infra-red spectra were measured on a Perkin–Elmer Infracord (model 137) spectrophotometer. Ultra-violet spectra were measured on a Beckmann DB spectrophotometer. The NMR spectra were made in a Varian A-60 Nuclear Magnetic Resonance spectrometer (tetramethylsilane as internal reference).

Determination of Neutral Equivalent

The neutral equivalent of pigments was determined by potentiometric titration with standard alkali. Allowance was made for the fact that betanin and amarantin titrate as dicarboxylic and tricarboxylic acids respectively, one carboxyl group being internally neutralized by a basic group.

Isolations

(*a*) *Pigments from* Phyllocactus hybridus *flowers.* A modification of the published procedure was used.[8] Flowers of *Phyllocactus hybridus* Hort. (3 kg) were ground under chilled methanol (2 l.) in a Waring blendor and the homogenate was filtered through cheese-cloth; the extraction was repeated two times with 70:30 (v/v) mixture of methanol–water (3 l.). The combined filtrates were concentrated *in vacuo*. The concentrated solution, cooled to 5°, was adjusted to pH 3 by adding 1 N HCl and clarified by centrifuging. The supernatant was passed onto a column of Dowex 50W-X2 (H^+ form, 20 × 5 cm) kept at 5°; the column was washed with 0·1% HCl (4 l.). Pigments were then eluted with water. The eluant was vacuum concentrated at 30° to a volume of about 100 ml. The concentrated solution was chromatographed in two portions on a 30 × 5 cm powdered polyamide column, cooled at 5°, using 5% aq. citric acid as the eluent. Two betacyanin fractions were obtained. *Fraction* 1, which emerged from the column after 1·4 l., contained betanin and isobetanin. *Fraction* 2, which emerged after 2·3 l., was freed from citric acid by resin treatment. On concentration to a small volume (10 ml) and standing overnight at 4°, it gave a crystalline mixture of phyllocactin and isophyllocactin (74 mg).

(*b*) *Pigments from* Celosia cristata *inflorescences.* In a typical run 1·5 kg of violet inflorescences of *Celosia cristata* L. was homogenized in a blendor under ice water (10 l.) and allowed to extract for 18 hr at 4°. The aqueous extract was filtered off through cheese-cloth from the solid residue, which was re-extracted with ice water (2 l.). From the combined extracts, adjusted to pH 3, a crude betacyanin mixture was obtained by chromatography on Dowex 50 W-X2 (H^+ form, 45 × 5 cm), as described above for the pigments of *Phyllocactus hybridus*. This mixture was chromatographed in four portions on polyamide column (18 × 5 cm, 5% aq. citric acid as developing solvent) and two fractions were obtained, which emerged from the column after 350 and 750 ml, respectively. They were further purified by resin treatment. *Fraction* 1, on concentration and standing overnight at 4°, gave a crystalline mixture of amarantin and isoamarantin (400 mg). *Fraction* 2 gave a crystalline celosianin–isocelosianin mixture (100 mg). A total of 300 mg of celosianin–isocelosianin from 4·5 kg of *Celosia cristata* inflorescences was obtained.

(*c*) *Pigments from* Iresine herbstii *leaves.* Fresh leaves of *Iresine herbstii* Hook (2 kg) were macerated with ice water (10 l.). From the filtered extracts, a crude betacyanin mixture was obtained by chromatography on two columns of Dowex 50W-X2 (H^+ form, 40 × 5 cm). This crude material was chromatographed in ten portions on polyamide powder column (16 × 5 cm). Elution with 5% aq. citric acid resulted in the separation of five fractions which emerged from the column after 300, 500, 900, 1300 and 2300 ml, respectively. These fractions, freed from citric acid by resin treatment, were separately concentrated to small volumes and kept overnight in the refrigerator. *Fraction* 1 yielded 120 mg of a crystalline mixture of amarantin and isoamarantin. *Fraction* 2 gave 550 mg of crystalline iresinin-I. *Fraction* 3, 4 and 5, which on standing did not give crystals, were vacuum evaporated to dryness thus yielding iresinin-II (90 mg), iresinin-III (12 mg) and iresinin-IV (27 mg), respectively.

Deacylation of Pigments and Identification of Constituents

Pigment (5–300 mg) was dissolved in deoxygenated water (2·5–150 ml) and hydrogen was passed through the solution for 5 min, after which 2 N NaOH (0·25–15 ml) was added and hydrogen bubbled through for another minute. The reaction mixture, whose colour slowly turned from red–violet to yellow, was allowed to stand under hydrogen for 3 hr at room temperature and then acidified with 2 N HCl, thus restoring the original red–violet colour. The resulting acidic solution was continuously extracted with ethyl acetate for 3 hr. The ethyl acetate extract, evaporated to dryness, gave a residue which was analysed for acids. 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.*

(*a*) *Identification of deacylated pigments.* Betanin–isobetanin was identified by (i) co-electrophoresis on paper with authentic samples at pH 2·4, 4·5 and 8·7, (ii) analytical column chromatography according to a previously described procedure,[1] (iii) hydrolysis with almond emulsin, and (iv) diazomethane methylation and subsequent alkali fusion, according to a described procedure.[4]

Amarantin–isoamarantin was identified by (i) co-electrophoresis on paper with authentic samples at pH 2·4, 4·5 and 8·7, (ii) analytical column chromatography, (iii) hydrolysis with β-glucuronidase and (iv) methylation with methyl iodide and silver oxide in dimethylformamide followed by acid hydrolysis and identification of the methylated sugars by PC and TLC, and GLC of the corresponding methyl glucosides.[7]

(*b*) *Purification and identification of acids.*

(i) *Malonic acid.* The acid obtained from phyllocactin–isophyllocactin was purified by band chromatography on Whatman 3 MM paper, using BAW as developing solvent. The band, R_f 0·66, located by a test strip spraying with bromophenol blue indicator, was excised and eluted with water. The eluate, acidified with conc. HCl, was extracted with ether. The ether extract was taken to dryness and in the residue malonic acid was identified by paper chromatography in BAW, PA and BPyW, thin-layer chromatography on silica-gel in BzAM and EAW2 and high voltage electrophoresis on paper at pH 1·8 for 90 min at 5000 V/90 cm in a Varsol tank. For additional confirmation the acid was converted, by treatment with excess ethereal diazomethane, in the corresponding methyl ester, which was subjected to GLC (column temp.: 119°). A single peak was observed with the sample alone or with a mixture of the sample and authentic dimethyl malonate.

(ii) 3-*Hydroxy*-3-*methylglutaric acid* (*HMG*). The acid from iresinin-I and isoiresinin-I was purified by preparative paper chromatography in BAW. The band, R_f 0·70, was cut out and eluted with water. The eluate, after acidification with conc. HCl, was extracted with

ether and the ether extract was evaporated to dryness. The residue, recrystallized from ether–light petroleum (b.p. 40–70°) mixture, furnished colourless flakes, m.p. 108°, undepressed on admixture with an authentic sample of HMG, prepared according to Adams and Van Duuren.[13] In PC it had the following R_f values: 0·70 in BAW and 0·45 in PA: in TLC on silica-gel 0·55 in BzAM. Its methyl ester, obtained by reaction with diazomethane, had i.r. and NMR spectra identical to those of synthetic dimethyl 3-hydroxy-3-methylglutarate, prepared from HMG by diazomethane methylation.

(iii) *Hydroxycinnamic acids.* They were isolated by chromatography in BPyW. The bands, revealed by examining the chromatograms in u.v. light in the presence of ammonia vapour, were cut out, eluted with 96% ethanol and the eluate further subjected to paper chromatography with BzAW1. Ultraviolet spectra of the so obtained hydroxycinnamic acids were taken against a solution provided by eluting with ethanol a piece of paper of same size and R_f as the original band from a blank chromatogram. Spectra were measured first in slightly acid solution (one drop of 1 N HCl was added to the eluates and the blanks), and then in alkaline solution (two drops of 2 N NaOH was added to the previous solutions). The *trans*-hydroxycinnamic acids were further characterized by R_f values in PC using six different solvent systems, and in TLC on silica-gel and polyamide powder.

Diazomethane Methylation of Pigments followed by Alkali Fusion

To a suspension of pigment (5 mg) in methanol (5 ml) an excess of ethereal solution of diazomethane was added. The mixture was kept at room temp for 1 hr and after removal of the solvents, to the residue 25% aq. NaOH (5 ml) was added and the solution refluxed for 15 min under nitrogen. After cooling, the solution, acidified with conc. HCl, was extracted with ether. The ether extract was analysed by TLC on silica-gel for indole derivatives. All the examined pigments gave 5-hydroxy-6-methoxyindole-2-carboxylic acid.

Enzymic Hydrolysis of Pigments

To a solution of pigment in sodium acetate buffer (pH 5), almond emulsin or β-glucuronidase (1 mg/10 mg of pigment) was added and the mixture kept at 37°; betanin and amarantin were used as a control of the activity of emulsin and β-glucuronidase respectively. Electrophoretic analysis (pH 2·4, 4·5 and 8·7) of the reaction mixture and an enzyme-free control solution were made at 15-min intervals during 3 hr, to test whether hydrolysis had taken place. In the case of iresinin-I a larger scale experiment was performed and, at the end of the hydrolysis, the resulting pigment (desglucuronosyliresinin-I) was recovered from the reaction mixture by absorption on Dowex 50W-X2 and elution with water. Desglucuronosylisoiresinin-I was analogously obtained from isoiresinin-I.

Acid Hydrolysis of Pigments

A solution of the pigment (1–30 mg) in 22% hydrochloric acid (0·5–15 ml) was heated at 80° for 5 min. The hydrolysate was evaporated to dryness *in vacuo* and excess HCl was removed by adding a small amount of water to the residue and evaporating to dryness four successive times. The residue was finally dissolved in water and the aglycones were characterized by E_b values in paper electrophoresis (pH 2·4, 4·5 and 8·7) and spectral determinations both in water and borate buffer pH 8·7. The remainder of the solution was applied to a small column of polyamide which was eluted with water. The eluate was concentrated to a small volume and analysed for carbohydrates and aliphatic acids by chromatography.

[13] R. ADAMS and B. J. VAN DUUREN, *J. Am. Chem. Soc.* **75**, 2377 (1953).

In the case of desglucuronosyliresinin-I and desglucuronosylisoiresinin-I, paper chromatographic analysis of the eluates from the nylon column showed the presence of a substance, which was separated from glucose and HMG by preparative paper chromatography in EaPyW. The band R_g 0·43, located with aniline phosphate, was eluted with water. The eluate, evaporated to dryness, gave a substance R_g 0·71 in BPyW and 1·70 in BAW, which was subjected to alkaline hydrolysis (0·2 N NaOH, 3 hr at room temp.). The solution was freed from alkali by passing through a column of Amberlite IRC-50 (H^+ form); the eluate was extracted with ether. In the aqueous solution glucose was identified; in the ether extract HMG was identified by PC and TLC.

In the case of celosianin–isocelosianin, separation and characterization of sugar esters, obtained by acid hydrolysis, required a modification of the procedure. The polyamide column (12 × 3 cm for 150 mg of pigment) was washed with water, thus removing glucose, glucuronic acid, glucuronolactone and sophorobiuronic acid, and then developed with water–methanol mixtures of increasing methanol concentrations. The 50% methanol eluate was chromatographed on Whatman 3 MM paper in BAW; four bands (A1, R_f 0·25; A2, R_f 0·30; A3, R_f 0·53 and A4, R_f 0·60) were located under u.v. light after exposure of the dried chromatogram to ammonia vapour. The 70% methanol eluate, when chromatographed in BAW, gave three bands, located as above (B1, R_f 0·66; B2, R_f 0·75 and B3, R_f 0·90). The different bands were separately extracted with 70% ethanol and the extracts examined by paper chromatography using a variety of solvents. All the bands gave a single spot, with the exception of B3, which was shown to be a mixture of *p*-coumaric and ferulic acids. Ultra-violet spectra of the other bands were measured and in every case a maximum ranging from 314 to 328 mμ was observed in acidic solutions and a maximum ranging from 352 to 365 mμ in alkaline solutions. Alkaline hydrolysis of the so obtained sugar esters was performed as above and the results are summarized in Table 1.

Hydrogen Peroxide Oxidation of Phyllocactin–Isophyllocactin

A solution of phyllocactin–isophyllocactin (10 mg) in 36% hydrogen peroxide (10 ml) was kept at room temp. for 48 hr. In order to decompose the excess hydrogen peroxide, to the resulting colourless solution palladium–charcoal (10 mg) was added. Catalyst was removed by filtration and the filtrate was concentrated *in vacuo*. Among the degradation products, glucose and malonic acid were identified, and a sugar ester which was isolated by band chromatography in BAW; upon alkaline hydrolysis, this ester yielded glucose and malonic acid.

Periodate Oxidation of Pigments

(*a*) *Phyllocactin–isophyllocactin and desglucuronsyliresinin-I.* To a solution of pigment (10 mg) in water (3 ml) a 1% $NaIO_4$ solution (2·6 ml) was added, the mixture kept in the dark at room temp. for 1 hr, $NaBH_4$ (18 mg) in water (3 ml) added, the solution kept at room temp. for 5 hr and then heated with HCl (2 N, 1·5 ml) at 60° for 20 min. After cooling, the solution was adjusted to pH 7 by adding 1 N NaOH, $NaBH_4$ (18 mg) in water (3 ml) added, the mixture kept at room temp. for 5 hr and then deionized on columns of Dowex 50W-X2 (H^+ form) and Dowex 21 K (OH^- form). The eluate was vacuum concentrated to 1 ml and analysed for polyols by PC.

(*b*) *Celosianin–isocelosianin.* Periodate oxidation of celosianin–isocelosianin and subsequent borohydride reduction and acid hydrolysis were performed as described above. At the end of the hydrolysis the solution was evaporated to dryness and the residue was dissolved

in water (1 ml) and examined by paper chromatography for carbohydrates and polyols. Additional confirmation on identity of glyceric acid and glucuronic acid was obtained by paper electrophoresis in phosphate buffer at pH 8.

Methylation with Methyl Iodide of Pigments

(*a*) *Phyllocactin–isophyllocactin and desglucuronosyliresinin-I.* Methylation procedure with methyl iodide in dimethylformamide in the presence of silver oxide, previously described for amarantin,[7] was used. The permethylated pigment was hydrolysed by refluxing for 4 hr with 0·3 N HCl and, after cooling, the solution taken to dryness *in vacuo*. The residue, dissolved in water, was analysed for methylated sugars by PC and TLC, and GLC (column temp. 158° or 169·5°) of their methyl glucosides. Both pigments gave 2,3,4-tri-*O*-methyl-D-glucose and 2,3,4,6-tetra-*O*-methyl-D-glucose; the formation of the latter compound indicates that, during methylation, removal of the acyl group also had occurred.

(*b*) *Iresinin-IV.* The pigment obtained by permethylation of iresinin-IV was hydrolysed with alkali (0·2 N NaOH) at room temp. for 3 hr. After acidification, the solution was extracted with ether and in the ether extract 3,4-dimethoxycinnamic acid and 3,4,5-trimethoxycinnamic acid were identified by TLC on silica-gel in BzAW2.

Isomerization of Iresinin-I and Isoiresinin-I

Iresinin-I and isoiresinin-I have been shown to be C-15 diastereoisomers by using a previously described procedure.[1]

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