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THE STABILIZING EFFECT OF THE ACYL GROUP ON THE CO-PIGMENTATION OF ACYLATED ANTHOCYANINS WITH C-GLUCOSYLFLAVONES

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Key Word Index—Acylated anthocyanin; flavocommelin; co-pigmentation constant; hydrophobic interaction; commelinin.

Abstract—The stability of the complex formed between acylated anthocyanins and flavocommelin was compared with that between unacylated anthocyanins and the flavone. At low anthocyanin concentrations (5×10^{-4} M), the complex involving acylated anthocyanins was much stabler than that involving ordinary anthocyanins. This extra stability is due to the acyl moiety in the acylated anthocyanins. The co-pigmentation constant (*Kc*) is defined as the affinity between an anthocyanin and its co-pigment.

INTRODUCTION

The basis of blue flower color in higher plants has been explained in terms of metal chelation of anthocyanins [1–4], co-pigmentation with flavone or tannin [5], or complexing with a macromolecule such as polysaccharide or polypeptide [6]. Typical blue flower pigments are protocyanin [7] or cyanocentaurin [8] from *Centaurea cyanus*, and commelinin [9] from *Commelina communis*. Asen *et al.* [10] reported that the degree of co-pigmentation of unacylated anthocyanin to flavone and related compounds was influenced by the concentration of both anthocyanin and co-pigment. Little is known of the effects of the acyl groups of anthocyanins on co-pigmentation. A few acylated pigments, e.g. those of iris [11] and larkspur [12], were investigated by Asen *et al.*, but details of the influence of the acyl group on co-pigmentation is obscure.

Commelinin is a sky-blue pigment reported by Takeda and Hayashi [13] to be composed of awobanin (a monoacylated anthocyanin), flavocommelin (a *C*-glucosylflavone), and magnesium (molar ratio 2:2:1). The extraordinary stability of this pigment was assumed by Hayashi *et al.* [14, 15] to be attributable to the chelate between magnesium and flavocommelin. Bayer [16] threw some doubt on the chelating stability of magnesium ion. Recently Goto *et al.* [17] demonstrated that magnesium was not necessary for the formation of the blue color of commelinin and they proposed that the color was due to a stacked molecular complex of the anthocyanin and the flavone. The origin of the stability and blueness of commelinin still remains obscure.

We now report that the acyl groups of anthocyanins have an important role in the stability and blueing effect of anthocyanin–flavonoid co-pigment complexes.

RESULTS AND DISCUSSION

Acylated anthocyanins which have a *p*-coumaroyl-glucose moiety at the 3-position of the anthocyanidin nucleus and their deacylated anthocyanins were used to examine the effect of the acyl group on co-pigmentation. Anthocyanins used in this experiment were: awobanin(delphinidin 3-*p*-coumaroyl glucoside-5-glucoside) (A), tibouchinin(malvidin 3-*p*-coumaroyl-glucoside-5-glucoside) (T), delphin(delphinidin 3,5-diglucoside) (D), delphinidin 3-monoglucoside (Dp-G), and malvin(malvidin 3,5-diglucoside) (M). Flavocommelin(4′-glucosyl-swertisin) (F) was utilized as the co-pigment.

Figure 1 shows the visible absorption spectra of TF and MF complexes measured 2 hr after dissolving in phosphate buffer (pH 6.0) at a variety of molar ratios of co-pigment to anthocyanin (conc. 5×10^{-4} M). Both malvidin-based anthocyanins, T and M, were stabilized with F, the acylated anthocyanin (T) being much more strongly stabilized than the other (M). In the presence of a large excess of co-pigment, both anthocyanins gave almost identical spectra, suggesting that while the acyl group in T has the ability to stabilize the complex it has almost no effect on color variation.

Figure 2 shows the maximal absorbance of TF or MF complexes against time after solution in phosphate buffer (pH 6.0). Rapid decolorization occurred in the absence of the co-pigment; M and T were almost colorless after 2 hr. Addition of the co-pigment F delayed conversion of the anhydrobase to the colorless pseudobase. T anhydrobase was stabilized to the same extent as M anhydrobase but at a concentration *ca* five times lower. Thus, the acyl group of T had a stabilizing effect on complex formation.

At a fixed concentration of anthocyanin (5×10^{-4} M), an increase in co-pigment concentration re-

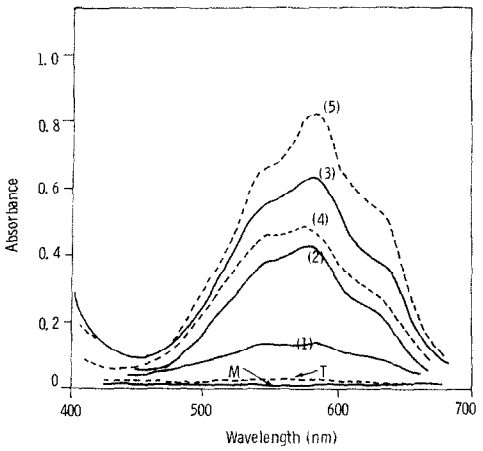

Fig. 1. Visible spectra of MF or TF complexes after 2 hr in phosphate buffer, pH 6.0 (anthocyanin concentration; 5×10^{-4} M, path length; 1 mm). Molar ratio of F to M (malvin) or T (tibouchinin) is as follows: —— MF complex, (1) 4.6:1, (2) 7.7:1, (3) 12.8:1; ---- TF complex, (4) 1.5:1, (5) 8.6:1.

sulted at first in a rapid rise in the visible absorbance (Fig. 3). As the co-pigment concentration continued to increase, however, the increase in absorbance slowed down and eventually no further change in absorbance was observed. From Fig. 3, the co-pigment equivalents required to yield half-maximal can be determined. This is defined as the co-pigmentation constant (*Kc*). The reciprocal, $1/Kc$, can be considered as an index of the affinity of an anthocyanin for its co-pigment. The *Kc* value changes with the concentration of anthocyanin, and *Kc* values, therefore, must be compared at the same molar concentration of anthocyanins. *Kc* values of TF and MF complexes were found to be 1.6 and 8.0, respectively, at 5×10^{-4} M anthocyanin. Thus, the *p*-coumaroyl residue in T increases the attraction between M and F five-fold. At a higher anthocyanin concentration (5×10^{-3} M), the MF complex gives a considerably lower *Kc* value (1.5), which is nearer to the value (1.1) of the TF complex. Thus, complex formation is much more dependent on the anthocyanin concentration in the case

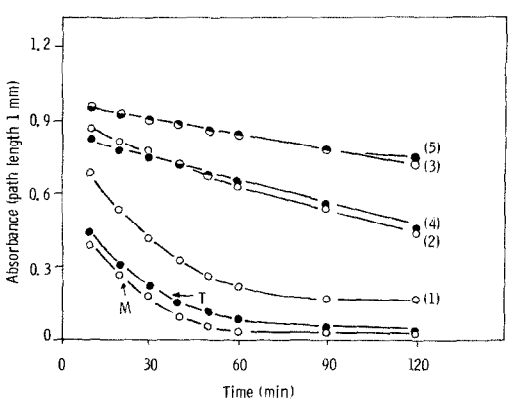

Fig. 2. Absorbance at λ_{max} of TF or MF complexes against time. Molar ratio of F to M or T is as follows: ○——○ MF complex, (1) 4.6:1, (2) 7.7:1, (3) 23.5:1; ●——● TF complex, (4) 1.5:1, (5) 4.6:1.

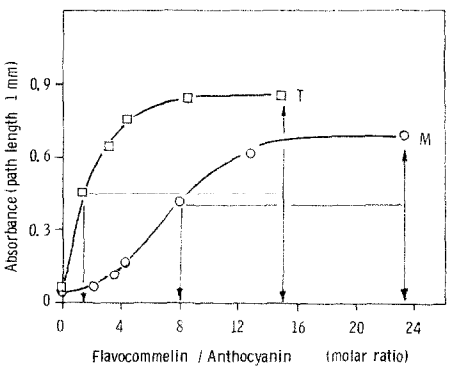

Fig. 3. Effect of flavocommelin concentration on absorbance of malvidin-based pigments (5×10^{-4} M) after 2 hr at pH 6.0.

of the unacylated complex (MF), than for the acylated anthocyanin complex (TF). Thus, the acyl moiety increases the strength of the complex formed and also suppresses its dissociation, even in dilute solution.

Similar experiments were carried out in the delphinidin series using A, D, and Dp-G in order to determine the stability of the commelinin complex. In the presence of F, A showed a clearly structured and intense absorption maximum in the visible region, which closely resembled that of natural commelinin in spite of the complete absence of magnesium ion (Fig. 4). The corresponding unacylated anthocyanins, D and Dp-G, formed only weak complexes with F and failed to produce structured absorption.

The *Kc* values of AF and DF complexes were 1.0 and 12.0, respectively, (Fig. 5) at an anthocyanin concentration of 5×10^{-4} M. This result shows that A has a much greater affinity for the co-pigment than D. This may be because flavocommelin, F is tailored by nature to fit perfectly with awobanin, A. Since the *Kc* value of delphin is similar to that of delphinidin 3-glucoside, the glucose moiety at the 5-position of D

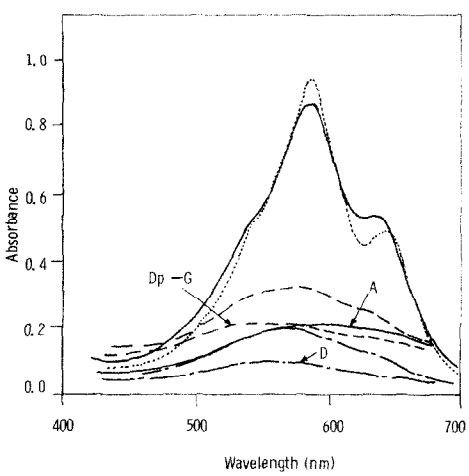

Fig. 4. Visible spectra of awobanin, delphin and delphinidin 3-monoglucoside complexed with flavocommelin after 2 hr at pH 6.0. Anthocyanin concentration 5×10^{-4} M; molar ratio of F to anthocyanin is in parentheses. —— AF complex (2:1), — — DF complex (4:1), — · — Dp-G·F complex (3.5:1), ------ natural commelinin (5×10^{-4} M as MW 1400 ([17]); path length 1 mm.

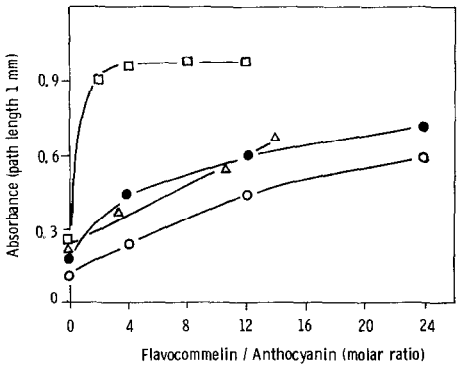

Fig. 5. Effect of flavocommelin concentration on absorbance of delphinidin-type compounds (5×10^{-4} M) after 2 hr at pH 6.0. □——□ A, ○——○ D, ●——● D+5×10^{-3} M $Mg(OAc)_2$, △——△ Dp-G.

has little effect on the stability of the complex [1]. Addition of magnesium acetate (5×10^{-3} M) stabilized the DF complex slightly, but produced a complex which was much less stable than the AF complex without magnesium ion. Thus, the *p*-coumaroyl group of A is important in stabilizing commelinin *in vivo*.

Gel filtration, which has been used for the purification of natural [14] and synthetic commelinin [17], was applied to these complexes. Malvin anhydrobase (10 mg) and F (molar ratio *ca* 1:2) were dissolved in a minimum amount of acetate buffer (pH 5.5), since the anthocyanin complex is stabler at a higher anthocyanin concentration. The solution was passed through a Sephadex G-15 column (0.8×12.5 cm); a purplish blue band was rapidly eluted with water, while excess starting materials remained on the column. This blue fraction was quickly dried *in vacuo*, and its composition determined by measuring its spectrum (Fig. 6) in a strongly acidic medium (*ca* pH 0.8). The spectrum was almost identical to the sum of the spectra of M and F (molar ratio 1:1) in the same solvent, indicating that

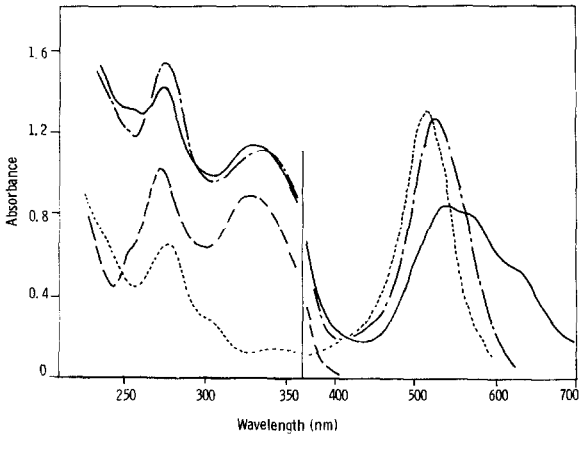

Fig. 6. UV-visible spectra of MF complex isolated by gel filtration. Path length 1 mm; —— MF in 4 M NaCl (concentration 6.3×10^{-4} M as MW 1300); — - — MF in 4 M NaCl containing 0.05 ml conc HCl; - - - - - - M in 0.2 N HCl–4 M NaCl (6×10^{-4} M); - - - F in 0.2 N HCl–4 M NaCl (6×10^{-4} M). In the ratio of absorption intensity at UV and visible region, isolated MF complex is almost identical to the mixture of M and F (molar ratio 1:1).

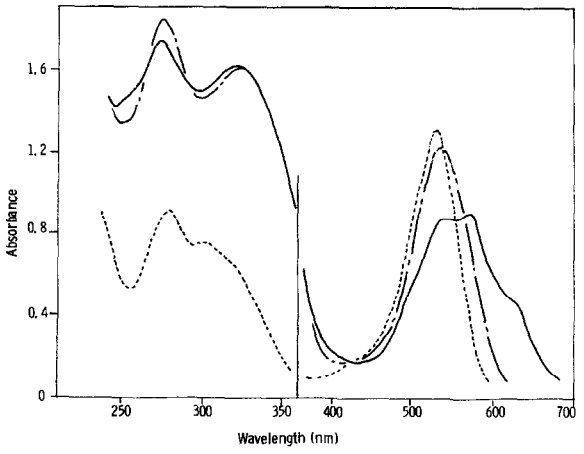

Fig. 7. UV-visible spectra of isolated TF complex. Path length 1 mm; —— TF in phosphate buffer, pH 6.0 (6×10^{-4} M as MW 1400); — - — TF in buffer (3 ml) containing 0.2 ml conc HCl, pH 0.8; - - - - T in HCl–KCl buffer, pH 0.8 (6×10^{-4} M).

the molar ratio of M and F in the blue complex is 1:1. This method was applied to TF and DF complexes (Figs. 7 and 8). In the case of DF, 4 M NaCl was used as the eluent in place of water, because the DF complex is otherwise too unstable; concentrated salt solutions stabilize the anhydrobases of anthocyanins [18]. The component ratio of the isolated TF complex by gel filtration was 1:1 (Fig. 7). Although the component ratio of the isolated DF complex was *ca* 1:1.5 (Fig. 8), a 1:1 ratio would presumably have resulted if the complex could have been further purified.

In a strongly acidic solution (pH 0.8), malvin and tibouchinin also form complexes with F, as indicated by their absorption maxima, which are shifted to longer wavelengths with increasing F concentration (Fig. 9). The shift with the acylated anthocyanin is larger than that with the unacylated one, at lower

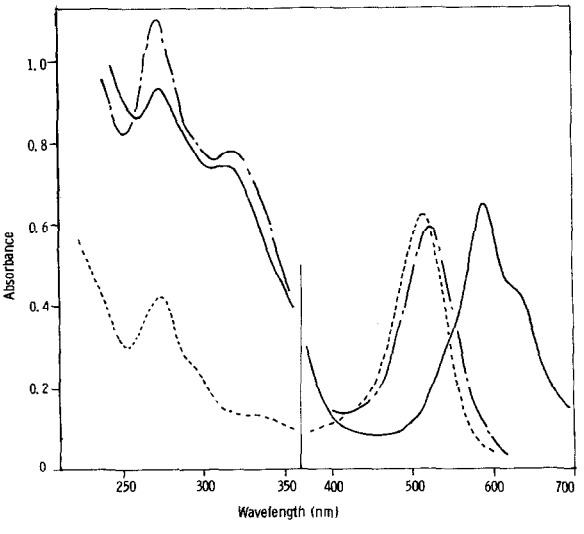

Fig. 8. UV-visible spectra of purified DF complex by gel filtration with 4 M NaCl. Path length 10 mm; —— DF in 4 M NaCl (concentration unknown); — - — DF in 4 M NaCl (3 ml) containing 0.1 ml conc HCl; - - - - - - D in 0.2 N HCl–4 M NaCl (2.8×10^{-5} M).

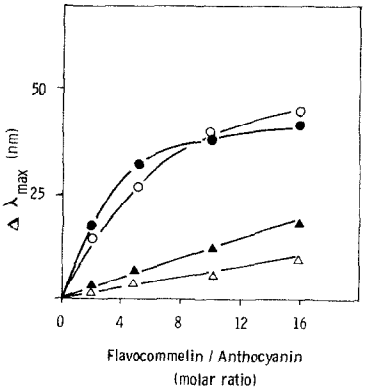

Fig. 9. Effect of flavocommelin on λ_{max} of anthocyanin in HCl–KCl buffer solution, pH 0.8. ○——○ M (5×10^{-4} M), △——△ M (2.5×10^{-5} M), ●——● T (5×10^{-4} M), ▲——▲ T (2.5×10^{-5} M).

molar ratios (<10) of F/anthocyanin. A similar effect is found in the case of awobanin and delphin. These facts indicate that the acyl moiety increases the size of the bathochromic shift shown by the co-pigment complex in strongly acidic media. Although the flavylium chlorides of M and T are difficult to dissolve at pH 0.8 to more than 5×10^{-4} M, addition of molar equivalent of the co-pigment causes them to go into solution immediately; thus the co-pigment can interact with the flavylium ion in acid solution (see also [11]).

In conclusion, the hydrophobic acyl group in awobanin and tibouchinin greatly stabilizes the molecular complex formed between anthocyanins and the flavone in a dilute aqueous solution. Goto *et al.* [17] suggested that commelinin is a 1:1 complex in which both anthocyanidin and flavone nuclei are stacked together, allowing maximum overlap of aromatic systems, and that the sugar moieties surrounding these aromatic systems (hydrophobic portion) make the complex relatively hydrophilic and water soluble. This concept of a stacked molecular complex is also supported by the evidence that the component ratio in the complexes isolated by gel filtration is 1:1. We suggest that the acylglucose moiety at the 3-position of the anthocyanidin links the flavone to the anthocyanin and with its hydrophobic nature, it tightens the bonding between the two flavonoid units.

EXPERIMENTAL

Materials. The purity of all isolated anthocyanins was examined spectrophotometrically. Tibouchinin chloride was isolated from the flowers of *Tibouchina semidecandra* [19]. The petals were immersed in HCl–MeOH (1 ml conc HCl/l.) at 4°. The soln was then subjected to filtration, cond *in vacuo* below 30° to dryness, dissolution in a small quantity of H_2O, and centrifugation to remove insoluble material. The supernatant was adsorbed on a polyamide column (3×25 cm), and then washed well with HCl–H_2O (1 ml conc HCl/l.) to remove impurities. Subsequently tibouchinin was eluted with HCl–MeOH (1 ml conc HCl/l.). Pure tibouchinin was finally purified by crystallization from MeOH–Et_2O. (Found: C, 50.95; H, 5.33. Calc. for $C_{38}H_{41}O_{19}Cl\cdot3H_2O$: C, 51.21; H, 5.28%). Malvin chloride was purchased from Aldrich. Awobanin chloride was obtained from commelinin. The soln of commelinin in 2 N HCl was allowed to stand at room temp., when the color gradually changed from blue to red. Separation of awobanin from the solution was carried out by use of Avicel microcrystalline cellulose column chromatography with HOAc–HCl–H_2O (5:1:40) as eluent. The anthocyanin fractions were evapd to min. vol. under red. pres. below 40° and then quickly dried over KOH. Delphin chloride was obtained after a soln of awobanin chloride dissolved in 20% aq. HCl was allowed to stand at room temp. for 1 week. The dark ppt. was collected by centrifugation and washed with EtOH. Crystallization was effected by dissolution in H_2O and addition of 7% HCl–EtOH. The absorption spectrum of the isolated anthocyanin (in 1% HCl–MeOH) lacked both an absorption band at 300–310 nm and a distinct shoulder in the 410–440 nm region. This is characteristic of an unacylated anthocyanin with a sugar at both the 3- and 5-positions [20]. (Found: C, 45.58; H, 5.13. Calc. for $C_{27}H_{31}O_{17}Cl\cdot3H_2O$: C, 45.25; H, 5.17%). FD MS:*m/e* 627 for $C_{27}H_{31}O_{17}^{+}$. Delphinidin 3-monoglucoside was isolated from blue hydrangea petals [21]. The petals were soaked overnight in HOAc and separated by filtration, and then the soln was evapd to dryness. The residue was dissolved in 3% HCl–MeOH and allowed to stand overnight when yellow ppts separated out. Filtration was carried out with aid of Celite. The purified anthocyanin was obtained by means of Avicel column chromatography with *n*-BuOH–HOAc–H_2O(4:1:5) as an eluent. Further purification was attained by use of HOAc–HCl–H_2O(15:1:84). Commelinin was isolated from the flowers of *Commelina communis* according to the method of Hayashi *et al.* [14, 22]. Flavocommelin was prepared according to the method of Takeda *et al.* [23].

The spectra of anthocyanin–co-pigment complexes. The anthocyanins were dissolved in MeOH, a few drops of 0.5 N NH_4OH were added to the soln to change the red flavylium ions to purple anhydrobases, and then the soln was quickly dried over P_2O_5 *in vacuo*. The weight of anhydrobase was measured on a microbalance. The soln of flavocommelin in 0.01 M Pi buffer was added to the previously weighed anhydrobase so that the concn of anthocyanins was 5×10^{-3} or 5×10^{-4} M. Absorption spectra were determined with a Hitachi 323 spectrophotometer at 23±2° using quartz cells having a path length of either 0.1, 1 or 10 mm which was employed at 5×10^{-3}, 5×10^{-4} or 2.5×10^{-5} M of anthocyanin concn, respectively.

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REFERENCES

1. Timberlake, C. F. and Bridle, P. (1975) in *The Flavonoids* (Harborne, J. B., Mabry, T. J. and Mabry, H., eds.) pp. 213–266. Chapman & Hall, London.
2. Shibata, K., Shibata, Y. and Kashiwagi, I. (1919) *J. Am. Chem. Soc.* **41,** 208.
3. Bayer, E. (1959) *Chem. Ber.* **92,** 1062.
4. Bayer, E., Nether, K. and Eggeter, H. (1960) *Chem. Ber.* **93,** 2871.
5. Robinson, G. M. and Robinson R. (1931) *Biochem. J.* **25,** 1687.
6. Robinson, R. and Robinson, G. M. (1939) *J. Am. Chem. Soc.* **61,** 1605.
7. Bayer, E. (1958) *Chem. Ber.* **91,** 1115.
8. Asen, S. and Jurd, L. (1967) *Phytochemistry* **6,** 577.
9. Hayashi, K., Abe, Y. and Mitsui, S. (1958) *Proc. Jpn. Acad.* **34,** 373.

10. Asen, S., Stewart, R. N. and Norris, K. H. (1972) *Phytochemistry* **11,** 1139.
11. Asen, S., Stewart, R. N., Norris, K. H. and Massie, D. R. (1970) *Phytochemistry* **9,** 619.
12. Asen, S., Stewart, R. N. and Norris, K. H. (1975) *Phytochemistry* **14,** 2677.
13. Takeda, K. and Hayashi, K. (1964) *Proc. Jpn. Acad.* **40,** 510.
14. Hayashi, K. and Takeda, K. (1970) *Proc. Jpn. Acad.* **46,** 535.
15. Takeda, K. and Hayashi, K. (1977) *Proc. Jpn. Acad.* **53,** 1.
16. Bayer, E. (1966) *Angew. Chem. Int. Ed. Engl.* **5,** 791.
17. Goto, T., Hoshino, T. and Takase, S. (1979) *Tetrahedron Letters* 2905.
18. Goto, T., Hoshino, T. and Ohba, M. (1976) *Agric. Biol. Chem.* **40,** 1593.
19. Harborne, J. B. (1964) *Phytochemistry* **3,** 151.
20. Harborne, J. B. (1958) *Biochem. J.* **70,** 22.
21. Lawrence, W. J. C., Price, J. R., Robinson, G. M. and Robinson, R. (1938) *Biochem. J.* **32,** 1661.
22. Mitsui, S., Hayashi, K. and Hattori, S. (1959) *Bot. Mag. Tokyo* **72,** 325.
23. Takeda, K., Mitsui, S. and Hayashi, K. (1966) *Bot. Mag. Tokyo* **79,** 578.