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POLYPRENYL DIPHOSPHATE SYNTHASE FROM MULBERRY LEAVES: STEREOCHEMISTRY OF HYDROGEN ELIMINATION IN THE PRENYLTRANSFERASE REACTION

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Key Word Index—*Morus bombysis*; Moraceae; mulberry; stereochemistry; prenyltransferase; polyprenyl diphosphate synthase; isopentenyl diphosphate; hydrogen elimination.

Abstract—A polyprenyltransferase catalysing the formation of *Z*-double bonds was partially purified from mulberry leaves, *Morus bombysis*. The enzyme catalysed a consecutive condensation of isopentenyl diphosphate with geranylgeranyl diphosphate as an allylic primer to produce a series of ficaprenol-type *Z,E*-mixed polyprenyl diphosphates with carbon chain length ranging from C_{40} to C_{60}. Not only *all-E*-geranylgeranyl diphosphate but also *E,E*-farnesyl- and geranyl diphosphates were accepted as substrates. Addition of Triton X-100 stimulated the enzymatic activity. The stereochemistry of hydrogen elimination from the 2-position of isopentenyl diphosphate during the *Z*-prenyl chain formation was examined directly by experiments using this synthase and (*S*)-[1-^{14}C, 2-^{3}H]isopentenyl diphosphate and it was demonstrated that the 2-pro-*S* hydrogen was lost. Feeding experiments of stereospecifically ^{3}H-labelled mevalonic acid with intact mulberry leaves, however, showed that mevalonic acid was incorporated into polyprenols with elimination of the 4-pro-*S* hydrogen of the acid.

INTRODUCTION

It was once generally accepted that the 4-pro-*S* hydrogen of mevalonic acid (MVA), which is equivalent to the pro-*R* hydrogen at C-2 of isopentenyl diphosphate (IPP), is lost in the formation of *E*-isoprene residues, whereas the 4-pro-*R* hydrogen (equivalent to the 2-pro-*S* hydrogen of IPP) is lost in the formation of *Z*-isoprene residues [1–3].

Hydrogen elimination in the formation of *Z*-isoprene residues was first studied in the biosynthesis of rubber by Archer *et al.* [2], who demonstrated that the 4-pro-*R* hydrogen of MVA was eliminated during the *Z*-isoprene residue formation. Examples of 4-pro-*R* hydrogen elimination were also reported in the biosynthesis of the *Z*-prenyl portions of hexahydroprenol in the mycelium of *Aspergillus fumigatus* [4] and of betulaprenols in the woody tissue of *Betula verrucosa* [5]. 4-Pro-*R* hydrogen elimination was also reported in the biosynthesis of the *Z*-isoprene residues of dolichols in rat liver [6]. *In vitro* experiments with partially purified prenyltransferases from *Lactobacillus plantarum* [7], *Micrococcus luteus* [8], *Bacillus subtilis* [9] and *Paracoccus denitrificans* [10] have directly demonstrated that the 2-pro-*S* hydrogen of IPP is eliminated during the enzyme reactions that form *Z*-prenyl chains. Recently, however, some papers have appeared reporting that *Z*-prenyl residues are formed in some higher plants with retention of the 4-pro-*R* hydrogen of MVA [11–15].

In order to refine stereochemical aspects of the prenyltransferase reaction that forms *Z*-prenyl residues, it is now necessary to obtain direct evidence for hydrogen elimination by using enzymes from higher plants. This paper describes the stereospecific removal of the 2-pro-*S* hydrogen of IPP during its incorporation into the *Z*-prenyl moiety of polyprenyl diphosphate with *Z,E*-mixed prenyl residues by the action of a prenyltransferase extracted from mulberry leaves, *Morus bombysis*.

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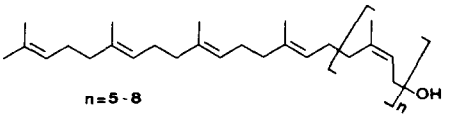
RESULTS

Detection of polyprenyl diphosphate synthase in the cell-free extract of mulberry leaves

It has been reported that mulberry leaves (*M. bombysis*) contain ficaprenol type polyprenols that consist of one dimethylallyl terminal unit (ω-terminal), three *E*-isoprene residues and a sequence of five to eight *Z*-isoprene residues aligned in that order [16]. In order to



obtain a cell-free system which contained prenyltransferase catalysing the formation of polyprenyl diphosphate with *Z*-prenyl residues of this type from IPP and an allylic diphosphate, we prepared a crude extract of mulberry leaves. As shown in Table 1, significant prenyltransferase activity was detected in the 108 000 *g* supernatant of the extract when allylic diphosphates were added as priming substrates. Moreover, the enzymatic activity was stimulated by Triton X-100, suggesting that the supernatant contained polyprenyl diphosphate synthase which, like undecaprenyl diphosphate synthase from bacteria [7–9], could be activated by detergent.

Table 1. Prenyltransferase activities in the 108 000 *g* supernatant of mulberry leaf extract

Allylic substrate	Activity/dpm *	Activity/dpm †
None	662	793
DMAPP	4174	12741
GPP	10182	18986
E,E-FPP	6216	13716
E,E,E-GGPP	2287	11914
Z,E,E-GGPP	977	11309

The enzymatic activities were assayed with [1-^{14}C]IPP and various allylic diphosphates in the presence or absence of Triton X-100 as described in *Experimental*.

* Assayed in the absence of Triton X-100.

† Assayed in the presence of Triton X-100.

Product analysis

The *n*-BuOH extracts from the reaction mixture of [1-^{14}C]IPP and *E,E*-farnesyl diphosphate (FPP) in the presence of Triton X-100 were analysed by radio-TLC. The radioactivity of the product was detected as a peak (R_f value, 0.46) between those of *all-E*-geranylgeranyl diphosphate (GGPP) and *all-E*-geranylgeranyl monophosphate, indicating that the radioactive product was polyprenyl diphosphate(s) with carbon chain-length longer than C_{20}.

The radio TLC chromatograms of the hydrolysate resulting from incubations of [1-^{14}C]IPP and geranyl diphosphate (GPP), FPP or GGPP in the presence of Triton X-100 showed a broad radioactivity peak over the R_f range of 0.6–0.8 in every case of incubation, indicating that the supernatant contained polyprenyl diphosphate synthase(s). For further analysis the polyprenols obtained by the reaction of [1-^{14}C]IPP and GGPP were subjected to radio-HPLC analysis. Five radioactivity peaks were observed at retention times of 9.5, 12.7, 16.4, 22.2, and 29.6 min. The major peak appeared at 16.4 min, which was slightly longer than that of *all-E*-decaprenol (15 min). The major polyprenol fraction obtained by a large-scale incubation was purified by HPLC and subjected to mass spectrometry. The polyprenol exhibited typical fragmentation patterns of an isoprenoid alcohol, showing an $[M]^+$ at *m/z* 698 ($C_{50}H_{82}O$) with fragment ions at *m/z* 680, 611, 543, 475, 407, 339, 271, 203, 135, and 69 (base peak). The mass spectrum and the chromatographic mobility described above indicate that the major product is decaprenyl diphosphate with *Z,E*-mixed stereochemistry of the ficaprenol type. Thus, it is reasonable to assume that the products synthesized by the incubation of IPP and GGPP in the presence of Triton X-100 are a series of similar *Z,E*-mixed type polyprenyl diphosphates with carbon chain lengths ranging from C_{40} to C_{60}. Even when GGPP was substituted with GPP or FPP, the polyprenol products gave radio-HPLC chromatograms similar to that obtained from GGPP. Hence, the polyprenyl diphosphate synthase activity found in the 108 000 *g* supernatant is responsible for the synthesis of the polyprenols found in mulberry leaves [16].

On the other hand, the enzymatic reaction product obtained by the incubation of [1-^{14}C]IPP and GPP in the absence of Triton X-100 was assigned as FPP, which was identified as *E,E*-farnesol after phosphatase treatment [17] followed by radio-TLC analysis (data not shown). This result indicates that FPP synthase is also extracted in the supernatant of mulberry leaf homogenate.

Seasonal variation in the level of prenyltransferase(s)

It has been reported that with increasing age of leaves there is a corresponding rise in the content of polyprenyl products in some higher plants [18, 19]. We examined the concentrations of prenyltransferases detectable in the 108 000 *g* supernatant of mulberry leaf homogenates. With maturing of the leaves a gradual increase in the amount of polyprenyl diphosphate synthase activity was observed (Fig. 1). On the contrary, FPP synthase activity decreased markedly in early summer. We therefore used mulberry leaves harvested in the middle of September as enzyme source.

Partial purification of polyprenyl diphosphate synthase

Because ammonium sulphate fractionation of the 108 000 *g* supernatant, which was viscous, resulted in a marked loss of the prenyltransferase activity, the supernatant was directly chromatographed on a DEAE-Toyopearl column. A fraction which showed prenyltransferase activity in the presence of Triton X-100 was obtained with *ca* 8% recovery of enzymatic activity (Fig. 2). Product analysis of the enzymatic reaction, however, showed that the fraction was still contaminated with FPP synthase. Further purification by hydroxylapatite or Sephadex G-100 chromatography was not successful.

Stereochemistry of hydrogen elimination at 2-position of IPP

The stereochemical course of the polyprenyl diphosphate synthase reaction was determined directly using the DEAE-Toyopearl purified enzyme or the 108 000 *g* supernatant with (*S*)-[1-^{14}C, 2-^{3}H]IPP and GGPP as substrates. Since pig liver FPP synthase is known to

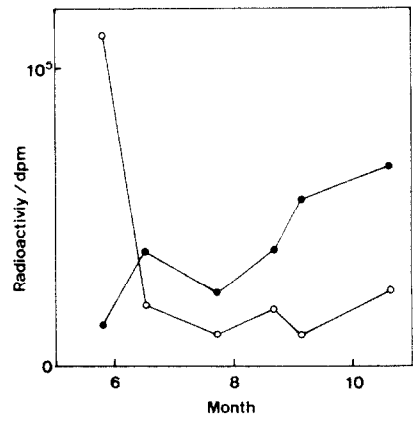


Fig. 1. Seasonal variation of the concentrations of prenyltransferases in mulberry leaves. ●, polyprenyl diphosphate synthase activity; ○, FPP synthase activity.

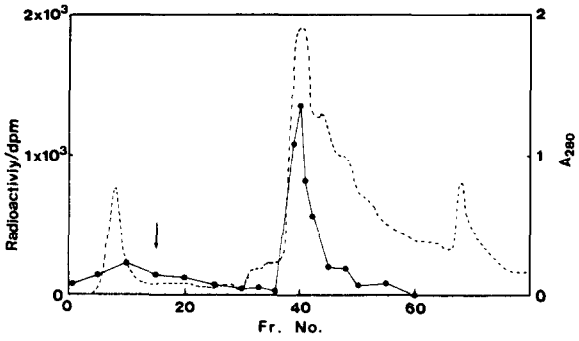


Fig. 2. DEAE-Toyopearl chromatography of mulberry leaf extract. Enzymatic activity was assayed as described in Experimental with [1-^{14}C]IPP and FPP in the presence of Triton X-100 (●——●). ----, A_{280}. The arrow indicates the starting point of gradient elution.

eliminate the 2-pro-*R* hydrogen of IPP during the formation of *E*-isoprene residues [1], the farnesol derived from the pig liver enzyme reaction was employed as a standard. All experiments showed that the 2-pro-*S* hydrogen of IPP is lost during the formation of a series of polyprenyl diphosphates of the ficaprenol type (Table 2). These results are in good agreement with the stereochemistry elucidated widely in the formation of polyprenyl chains [1–10], and hence are in conflict with the opposite results obtained recently from data on the incorporation of stereospecifically ^{3}H-labelled MVA into *Z*-prenyl chains in some higher plant leaves [11–13, 15].

Incorporation of (3R,4R)-[2-^{14}*C*, 4-^{3}H]MVA *into polyprenols in intact mulberry leaves*

In an attempt to resolve the reason for the conflicting results, we carried out feeding experiments of (3*R*,4*R*)-[2-^{14}C,4-^{3}H]MVA using mulberry leaves essentially according to the method reported by Suga *et al.* [15]. Labelled MVA was fed to the leaves and the hexane extracts were saponified. The polyprenol fractions were purified by TLC followed by HPLC. As shown in Table 3, the 4-pro-*R* hydrogen in MVA appeared to be retained during the *in vivo* formation of polyprenols in intact mulberry leaves. These results are apparently contradictory to those obtained by the *in vitro* experiments with a partially purified polyprenyl diphosphate synthase fraction.

DISCUSSION

A polyprenyl diphosphate synthase was partially purified from mulberry leaves and shown to catalyse the synthesis of *Z,E*-mixed type polyprenyl diphosphates with carbon chain length ranging from C_{40} to C_{60}. The synthase required Triton X-100 for enzyme activity similar to bacterial *Z,E*-mixed-polyprenyl diphosphate synthases so far investigated [7–10].

It was also confirmed that the 2-pro-*S* hydrogen of IPP was lost in the formation of the *Z*-isoprene residues of *Z–E*-mixed polyprenyl diphosphate. Although no experiments were conducted with the (*R*)-[1-^{14}C,2-^{3}H]IPP to obtain complementary results, the conclusion is reliable since our experiments showed that the polyprenols were formed from (*S*)-[1-^{14}C,2-^{3}H]IPP with loss of the ^{3}H, which was ascertained to be retained in the formation of *E,E*-farnesol by pig liver FPP synthase.

It is noteworthy that the stereochemistry in the case of the *M. bombysis* enzyme is opposite to that reported by Suga *et al.*, who showed that the 2-pro-*R* hydrogen of IPP was eliminated in the formation of the *Z*-prenyl moiety of similar polyprenols by the *Mallotus japonicus* enzyme [12, 15].

The feeding experiment with MVA, however, resulted in specific incorporation of the 4-pro-*R* hydrogen into the ficaprenol-type polyprenols in mulberry leaves. This result is in accord with the experiments reported by Suga *et al.* with leaves of a number of higher plants [15].

The discrepancy between the results obtained in our *in vivo* and *in vitro* experiments with *M. bombysis* could be

Table 2. Incorporation of (2*S*)-[1-^{14}C,2-^{3}H]IPP into polyprenols by the action of polyprenyl diphosphate synthase from mulberry leaves

Experiment		^{3}H (dpm)	^{14}C (dpm)	^{3}H/^{14}C
I	*E,E*-Farnesol*	61652	24145	2.55
	Z,E-mixed-Decaprenol (C_{50})†	4293	24159	0.18
	Z,E-mixed-Decarpenol (C_{50})‡	2125	25992	0.08
II	*E,E*-Farnesol*	78502	13352	5.88
	Z,E-mixed-Polyprenols§ (C_{45}, C_{50})	253	3096	0.08

* *E,E*-Farnesol was synthesized by pig liver FPP synthase reaction followed by alkaline phosphatase treatment.

† The reaction was carried out in the absence of iodoacetamide with the 108 000 *g* supernatant of mulberry leaf homogenate.

‡ The reaction was carried out in the presence of iodoacetamide with the 108 000 *g* supernatant.

§ The reaction was carried out with the DEAE-Toyopearl purified enzyme in the absence of iodoacetamide. The polyprenols were purified by TLC and analysed for radioactivity.

Table 3. Incorporation of (4*R*)-[2-^{14}C, 4-^{3}H]MVA into polyprenols in intact mulberry leaves

	^{3}H (dpm)	^{14}C (dpm)	^{3}H/^{14}C	Theoretical* ^{3}H/^{14}C
(4*R*)-[2-^{14}C, 4-^{3}H]MVA	99 459	45 887	2.16	
Decaprenol (C_{50})	3 815	1 854	2.06	0.87
Undecaprenol (C_{55})	4 220	2 221	1.90	0.79
Dodecaprenol (C_{60})	3 165	1 650	1.92	0.72

Details on the feeding experiments are described in *Experimental.*

* Calculated ratio based on the assumption that the (*E*)- and the (*Z*)-prenyl units in the polyprenol products are synthesized with elimination of the 4-pro-*S* and 4-pro-*R* hydrogen atoms of MVA, respectively.

attributed to compartmentalization of the polyprenyl diphosphate synthase that is responsible for the *Z*-prenyl chain elongation. In intact leaves the MVA fed externally could be metabolized to GGPP by a series of isoprenoid enzymes occurring in the cytosol. The GGPP thus formed could be transferred, because of its amphipathic properties, inside organelles where the *Z*-prenyl chain elongation is carried out with endogenous IPP, to which the organelle membrane is impermeable as well as to MVA. It has been reported that most of the *Z,E*-mixed polyprenols of plant leaves are found in the chloroplasts [20]. Moreover, MVA and its phosphate ester metabolites were impermeable to isolated spinach chloroplasts [21]. These facts support the assumption described above.

EXPERIMENTAL

Materials. Mulberry leaves (*M. bombysis* K.) were obtained locally in the middle of September, 1985 and kept frozen at −80° until used. Leaves for the study of seasonal variations in enzymes were collected from the same *M. bombysis* tree in the middle of every month from May to November. [1-^{14}C]IPP, GPP, FPP, and GGPP were the same preparations as used in a previous study [10]. (3*RS*,4*R*)-[4-^{3}H]MVA and [2-^{14}C]MVA were obtained from Amersham.

Extraction and partial purification of polyprenyl diphosphate synthase. Mulberry leaves (20 g) were ground at 4° for 30 min in a mortar with 20 g of sand in 100 ml of 100 mM K-Pi buffer, pH 7 containing 20 mM 2-mercaptoethanol and 1 mM EDTA. After filtration through two layers of gauze, the filtrate was centrifuged at 1400 *g* for 30 min. The supernatant was further centrifuged at 108 000 *g* for 60 min to give a viscous supernatant. The resulting supernatant (68 ml) was dialysed against 50 mM K-Pi buffer, pH 7 containing 10 mM 2-mercaptoethanol and 0.5 mM EDTA (Buffer A) overnight. The dialysate was applied to a 2.8 × 30 cm column of DEAE-Toyopearl which had been equilibrated with Buffer A. Elution was performed with a linear gradient of NaCl from 0 to 0.50 M in Buffer A (total vol. 1 l). Fractions containing 15 ml were collected (Fig. 2).

Assay for polyprenyl diphosphate synthase. The standard incubation mixt contained, in a final vol. of 1 ml, 100 μmol of K-Pi buffer, pH 7, 5 μmol $MgCl_2$, 0.5 mg of Triton X-100, 5 μmol of KF, 50 nmol of GGPP or FPP, 50 nmol of [1-^{14}C]IPP (sp. act. 1 Ci/mol), and a suitable amount of enzyme. After incubation at 30° for 14 hr, 0.2 ml of 6 M NaOH was added to stop the enzymatic reaction. The mixt was washed × 3 with 3 ml of Et_2O to remove the alcohols derived from the substrates by some phosphatases that coexisted in the enzyme soln. Then the enzymatic reaction products were extracted with 3 ml of *n*-BuOH after addition of *ca* 1 g of $(NH_4)_2SO_4$. The BuOH exts were back-washed × 3 with H_2O and a portion of the BuOH layer taken for radioactive counting. Enzyme activity is expressed as the radioactivity in the exts.

In the case of the assay for FPP and GGPP synthases, incubation was carried out with GPP and FPP as allylic substrates, respectively, without the addition of Triton X-100.

Analysis of enzymatic reaction products. Polyprenyl diphosphates were extracted with *n*-BuOH from the reaction mixture and hydrolysed enzymatically according to the method of ref. [17].

Extracts of polyprenyl diphosphates were analysed by silica gel TLC in *n*-PrOH–NH_3–H_2O (6: 3: 1).

Polyprenol exts were chromatographed by silica gel TLC in C_6H_6–EtOAc (9:1). Polyprenols were also analysed by radio-HPLC on a Hitachi porus polymer gel No. 3011 column (2.6 × 500 mm) with a solvent system of MeOH–hexane (4 : 1) at a flow rate of 0.8 ml/min. The elution of prenols was monitored by recording both A_{215} and radioactivity.

The major polyprenol products purified by TLC followed by HPLC under conditions similar to those described above were subjected to MS. Spectra were recorded at 187° with an accelerating voltage of 70 eV.

Synthesis of (2S)-[1-^{14}C,2-^{3}-H]*IPP.* (2*S*)-[2-^{3}H]IPP was synthesized enzymatically from (3*R*,4*R*)-[4-^{3}H]MVA (sp. act. 1.8 Ci/mmol) essentially according to the method of ref. [22]. The ^{3}H-labelled IPP was mixed with [1-^{14}C]IPP in an appropriate ratio as described later.

Stereochemical analysis of polyprenyl diphosphate synthase reaction. The incubation mixture contained, in a final vol. of 4 ml, 400 μmol of K-Pi buffer, pH 7, 20 μmol of $MgCl_2$, 2 mg of Triton X-100, 20 μmol of KF, 300 nmol of GGPP, 30.2 nmol of (2*S*)-[1-^{14}C,2-^{3}H]IPP (^{3}H/^{14}C = 2.55 as determined by enzymatic conversion to *E,E*-farnesol) and the 108 000 *g* supernatant of mulberry leaf homegenate (3.5 mg protein) (Expt I, Table 2). When partially purified enzyme was used, 42.4 nmol of (2*S*)-[1-^{14}C,2-^{3}H]IPP (^{3}H/^{14}C = 5.88 as determined as described above) was added (Expt II, Table 2). After incubation at 30° for 14 hr, each reaction mixture was treated by the procedure described for enzyme assay. Polyprenols were analysed by radio-HPLC or radio-TLC.

Feeding of (3R,4R)-[2-^{14}C,4-^{3}H]*MVA to leaves.* A mixt of (3*RS*,4*R*)-[4-^{3}H]MVA (0.80 μCi, sp. act. 1.8 Ci/mmol) and (3*RS*)-[2-^{14}C]MVA (0.37 μCi, sp. act., 45.5 Ci/mol) were fed to mulberry leaves (fr wt. 20 g) through their petioles over 2 hr at room temp. After uptake of tracer, H_2O was fed over 70 hr. The leaves were then cut into small pieces and treated × 3 with 200 ml portions of MeOH. The MeOH extracts were concd to *ca* 50 ml and then extracted × 4 with 40 ml of hexane. The hexane layer was collected and concd to *ca* 20 ml. After backwashing with H_2O, solvent was removed on a rotary evaporator and the resulting residue dissolved in 4 ml of 7.5% (w/v) KOH in EtOH. The soln was heated at 100° for 20 min and then extd × 3 with 5 ml portions of pentane. Polyprenols were purified by silica gel TLC followed by HPLC and their ^{3}H/^{14}C ratios measured.

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REFERENCES

1. Cornforth, J. W., Cornforth, R. H., Donninger, C. and Popják, G. (1966) *Proc. R. Soc. Ser. B*, **163**, 492.
2. Archer, B. L., Barnard, D., Cockbain, E. G., Cornforth, J. W., Cornforth, R. H. and Popják, G. (1966) *Proc. R. Soc. Ser. B*, **163**, 519.
3. Poulter, C. D. and Rilling, H. C. (1981) in *Biosynthesis of Isoprenoid Compounds* (Porter, J. W. and Spurgeon, S. L., eds) Vol. 1, p. 162. Wiley, New York.
4. Stone, K. J. and Hemming, F. W. (1967) *Biochem. J.* **104**, 43.
5. Gough, D. P. and Hemming, F. W. (1970) *Biochem. J.* **117**, 309.
6. Gough, D. P. and Hemming, F. W. (1970) *Biochem. J.* **118**, 163.
7. Allen, C. M., Keenan, M. V. and Sack, J. (1976) *Arch. Biochem. Biophys.* **175**, 236.
8. Baba, T. and Allen, C. M. (1978) *Biochemistry* **17**, 5598.
9. Takahashi, I. and Ogura, K. (1982) *J. Biochem. (Tokyo)* **92**, 1527.

10. Ishii, K. Sagami, H. and Ogura, K. (1986) *Biochem. J.* **233**, 773.
11. Jedlicki, E., Jacob, G., Faini, F., Cori, O. and Bunton, C. A. (1972) *Arch. Biochem. Biophys.* **152**, 590.
12. Suga, T., Hirata, T., Aoki, T. and Shishibori, T. (1983) *J. Am. Chem. Soc.* **105**, 6178.
13. Suga, T., Aoki, T., Hirata, T. and Saragai, Y. (1983) *Chem. Letters* 1467.
14. Banthorpe, D. V., Bunton, C. A., Cori, O. and Francis, M. J. O. (1985) *Phytochemistry* **24**, 251.
15. Suga, T., Hirata, T., Aoki, T. and Kataoka, T. (1986) *J. Am. Chem. Soc.* **108**, 2366.
16. Toyoda, M., Fukawa, H. and Shimizu, T. (1969) *Nippon Nogeikagaku Kaishi* (in Japanese) **43**, 688.
17. Koyama, T., Fujii, H. and Ogura, K. (1985) *Methods Enzymol.* **110**, 153.
18. Wellburn, A. R. and Hemming, F. W. (1966) *Phytochemistry* **5**, 969.
19. Ibata, K. Mizuno, M., Takigawa, T. and Tanaka, Y. (1983) *Biochem. J.* **213**, 305.
20. Hemming, F. W. (1967) in *Terpenoids in Plants* (Pridham, J. B. ed.), p. 223. Academic Press, London.
21. Kreuz, K. and Kleinig, H. (1981) *Planta* **153**, 578.
22. Popják, G. (1967) *Methods Enzymol.* **15**, 393.