Induction of Phytoalexin Synthesis in Soybean. Structure and Reactions of Naturally Occurring and Enzymatically Prepared Prenylated Pterocarpans from Elicitor-Treated Cotyledons and Cell Cultures of Soybean

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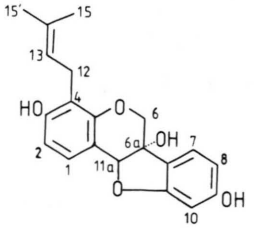
Using HPLC on a partisil column (5 µm) it was possible to separate two reaction products from an enzyme incubation of dimethylallylpyrophosphate and 3,6a,9-trihydtoxypterocarpan with a particulate fraction from elicitor-treated soybean cotyledons (Harosoy 63) or soybean cell cultures. These two products were identified by MS and ^{1}H-NMR analysis as 2- and 4-dimethylallyltrihydroxypterocarpan. Both compounds also occur as natural products in elicitor-treated soybean cotyledons and to a smaller extent in soybean cell cultures. Introduction of the dimethylallyl substituent into trihydroxypterocarpan increases the fungitoxicity against *Cladosporium cucumerinum*.

Upon treatment with alkali, solutions of 3,6a,9-trihydroxypterocarpans turn to an intense red colour with λ_{max} around 530 nm. One of the products from 2-DMA-THP could be isolated by reversed phase HPLC. According to its mass spectrum a quinoid structure is proposed for this red pigment.

Introduction

Upon infection with *Phytophtora megasperma* f. sp. *glycinea* [1] or upon induction with a glucan derived from cell walls of this fungus (Pms-elicitor) [2] soybean seedlings (*Glycine max* L.) accumulate pterocarpanoid phytoalexins. These phytoalexins have been identified as the glyceollin isomers I–IV [2, 3] and as 3,6a,9-trihydroxypterocarpan (**1**) [3,4] (Fig. 1). Phytoalexin accumulation can also be induced in cell suspension cultures of soybean [5]. Concomitant with accumulation of phytoalexins an increase in the activities of enzymes involved in flavonoid biosynthesis was found [6, 7].

We recently reported the presence of a dimethylallyl-pyrophosphate:3,6a,9-trihydroxypterocarpan dimethylallyl transferase in a particulate fraction from elicitor-treated soybean cotyledons [8, 9]. This enzyme which cannot be detected in wounded cotyledons, is supposed to catalyse a key step in biosynthesis of glyceollins. The enzymatic product was identified as 2-dimethylallyl-3,6a,9-trihydroxypterocarpan (**3**) [7]. HPLC analysis indicated a second minor compound which was tentatively identified as 4-DMA-THP (**2**). We now report the unequivocal identification of 2- and 4-dimethylallyl-trihydroxypterocarpan as products of various enzyme preparations from induced soybean cotyledons and soybean cell suspension cultures. These two isomers also occur as natural products in elicitor-treated cotyledons and cell cultures. Furthermore we propose a structure for one of the red pigments formed upon alkaline treatment of 2-DMA-THP.



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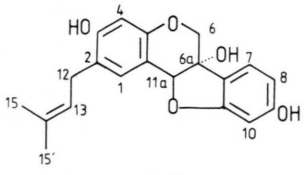

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Abbreviations: DMA, dimethylallyl; DMAPP, dimethylallyl-pyrophosphate; THP, (6aS, 11aS)3,6a,9-trihydroxypterocarpan; HPLC, high pressure liquid chromatography; HPTLC, high performance thin layer chromatography; Pms, *Phytophthora megasperma* f. sp. *glycinea* (syn. f. sp. *sojae*).

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Materials and Methods

Chemicals

[1-^{14}C]Isopentenylpyrophosphate (57 Ci/mol) was obtained from Amersham Buchler. Its conversion to [1-^{14}C]dimethylallylpyrophosphate and the isolation of 3,6a,9-trihydroxypterocarpan have been described previously [8, 9]. 3,9-dihydroxypterocarpan was a gift from Dr. P. M. Dewick, Nottingham, U. K.

Plant material and induction

The isolation of the Pms-elicitor and induction of soybean cotyledons (*Glycine max* L. cv. Harosoy 63) was carried out as described in [6].

Cell suspension cultures obtained from Harosoy 63 hypocotyls were cultivated in B5-medium [5] and transferred every 5–6 days. For induction Pms-elicitor dissolved in 10 mmol/l phosphate buffer, pH 7.2, was added to a final concentration of 125 µg/ml. The cells were harvested 10–20 h after addition of elicitor.

Prenyltransferase preparations

a) from cotyledons

The 48000 × *g* pellet from elicitor-treated cotyledons was prepared according to [8]. Further purification of the transferase preparation by sucrose gradient centrifugation and solubilization will be described elsewhere [10].

b) from cell cultures

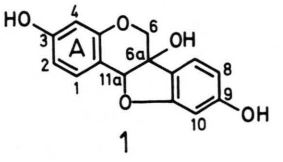
The induced cells (80–100 g fresh wt) were collected on a nylon net (mesh size 20 µm) in a Büchner funnel and resuspended in 120–150 ml of the following buffer: 0.2 mol/l Tris-HCl, pH 7.5, 1 mmol/l EDTA, 7 mmol/l 2-mercaptoethanol and 20% (w/w) saccharose. The suspension was homogenized in a Potter-Elvehjem homogenizer at 500–750 rpm at 0–2 °C and the homogenate centrifuged at 110000 × *g* for 60 min. The pellet was resuspended in a small volume of the extraction buffer and this suspension was used for enzymatic incubations.

Prenyltransferase assay

The incubation mixture contained in a total volume of 0.1 ml: 10 µmol Tris-HCl, pH 7.7, 1.44 nmol [1-^{14}C]DMAPP (5.5 × 10^4 dpm), 100 nmol 3,6a,9-trihydroxypterocarpan in 2.5 µl ethyleneglycolmonomethylether, 3 µmol NaF, 1.2 µmol $MnCl_2$, 1.2 µmol glutathione (reduced form), and 5–20 µl of protein. After incubation for 60 min at 30 °C and addition of NaCl the mixture was extracted 3 times with 1 ml ethylacetate. An aliquot of the combined organic phases (100 µl) was counted for radioactivity.

Isolation of enzymatic and plant products

For preparative isolation of the prenylated products the ethylacetate extracts of several enzyme incubation were mixed with an ethylacetate extract from 500 elicitor-treated cotyledons. The combined extracts were concentrated at 20 °C with a stream of nitrogen and the concentrate applied to preparative silica gel plates (1 mm, silica gel G after Stahl, Merck). The plates were developed with solvent system 1 and scanned for radioactivity. The radioactive zone ($R_f \sim 0.4$) was scraped off and eluted with ethanol. The eluate was chromatographed on a Sephadex LH-20 column (3 × 28 cm) with methanol



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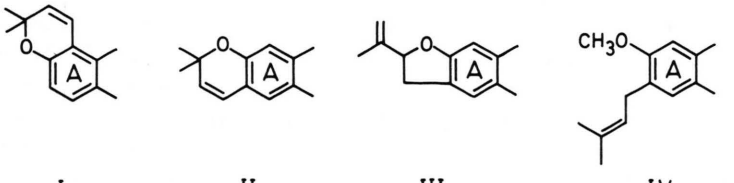

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Fig. 1. Structure of (6aS, 11aS)-3,6a,9-trihydroxypterocarpan (**1**) and of glyceollin isomers I–IV.

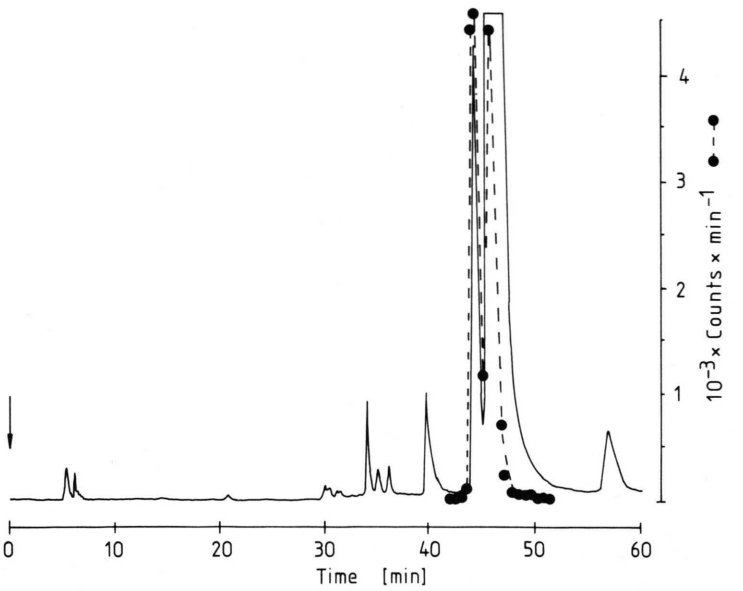

Fig. 2. Separation of the transferase incubation supplemented with ethylacetate extract of induced cotyledons by HPLC on a 5 µm Partisil column. The major product represents 97% and the minor product 3% of the mixture. —, absorbance at 280 nm.

and the absorbance at 280 nm recorded. The concentrated radioactive fractions were subjected to HPLC on a RP-18 column (0.9 × 25 cm) in methanol/water 75:25 v/v). Fractions with absorbance at 280 nm were collected and measured for radioactivity.

Separation of the 2- and 4-DMA-THP isomers was achieved on a 5 µm Partisil column (0.9 × 25 cm) with *n*-hexane/isopropanol (94:6 v/v). Rechromatography with *n*-hexane/isopropanol (95:5 v/v) gave the pure isomers.

Preparation of red pigment

100 µg 2-DMA-THP was dissolved in 1 ml ethanol. After addition of 5 µl 4 N KOH the solution was kept at 20 °C for 15 min, after which the red colouration (λ_{max} 534 nm) did not increase further. The solution was concentrated to 50 µl and then subjected to HPLC on a RP-18 column (0.9 × 25 cm) with methanol/water (75:25 v/v).

Analytical methods

Analytical TLC was performed on silica gel plates (0.25 mm, Merck F_{254}). For HPTLC nano plates (Machery & Nagel) were used. Solvent systems for both methods were: (1) toluene/chloroform/acetone (45:25:35, v/v/v); (2) benzene/acetic acid/water (125:72:3, v/v/v); (3) benzene/methanol (95:5, v/v).

For HPLC (Waters Associates) the following columns were used: a) for reversed phase chromatography LiChrosorb RP-18 (10 µm, Merck); b) for adsorption chromatography Partisil (5 µm, Whatman), which was determined to have 13000 theoretical plates. Mass spectrometry was performed on a Finnigan 4023 instrument. For NMR-analysis a Bruker WM 250 instrument was used.

Results

Identification of two products in the transferase reaction

Dimethylallyl transferases were found in elicitor-treated cotyledons as well as in cell suspension cultures of soybean. The activity was shown to be in a 105000 × *g* particulate fraction. The properties of the membrane-bound and solubilized transferases will be reported elsewhere [10]. Using the radioactive transfer product as marker the prenylated trihydroxypterocarpan could also be isolated from ethylacetate extracts of elicitor-treated soybean cotyledons [9]. It became now possible to investigate the question whether more than one transfer product is formed. Analysis of the transferase incubations by TLC, two-dimensional HPTLC with solvent systems 2 and 3 and by reversed phase HPLC on LiChrosorb RP-18 showed the presence of only one radioactive product. However, a clear separation into two pro-

ducts was achieved by HPLC on a 5 µm Partisil column (Fig. 2). By rechromatography of the individual products on Partisil, pure compounds were obtained which were used for MS and ^{1}H-NMR analysis. From 500 cotyledons treated with elicitor for 20 h 1.6 mg of the major and 50 µg of the minor product were isolated. This amount represents 1–2% of the total pterocarpan mixture. The major product (Fig. 2) gave mass and ^{1}H-NMR-spectra identical with those of 2-dimethylallyl-3,6a,9-trihydroxypterocarpan (**3**) [8].

From the MS of the minor compound with a molecular ion at *m/e* 340 (20%) and a very similar fragment ion pattern an isomeric structure could be assumed. The fragments of both products are listed in Table I. A comparison shows that the base peak of the minor compound is one mass unit lower than that of the 2-DMA-trihydroxypterocarpan. Assuming that the minor product is the 4-DMA isomer **2** this different fragmentation can be explained by the reaction sequence shown in Fig. 3. With both isomers one molecule of water is lost in a first step leading to a pterocarp-6a-ene structure with *m/e* 322. In the 4-isomer loss of an isobutene moiety is observed next, giving the base peak at *m/e* 266. This could be explained by a rearrangement reaction in which the hydrogen of the 3-OH group is transferred to the DMA-substituent in a 6-membered transition state [11, 19, 23]. On the other hand loss of methyl from the pterocarp-6a-ene intermediate through a complex rearrangement gives a fragment ion at *m/e* 307 [20]. The pterocarp-6a-ene fragment of the 2-DMA-isomer loses an isobutene radical leaving a benzylium ion which can undergo ring expansion to a tropylium cation with *m/e* 267 [8]. A minor fragmentation path is loss of OH· and formation of a second tropylium cation at *m/e* 305 [21].

Table I. Major mass spectral fragments of the 2- and 4-dimethylallyl-3,6a,9-trihydroxypterocarpans.

2-DMA-THP		4-DMA-THP	
m/e	Relative intensity [%]	*m/e*	Relative intensity [%]
340 (M^+)	20	340 (M^+)	20
322	98	322	63
321	26	321	8
305	7	307	21
268	19	267	25
267	100	266	100
266	50	265	37

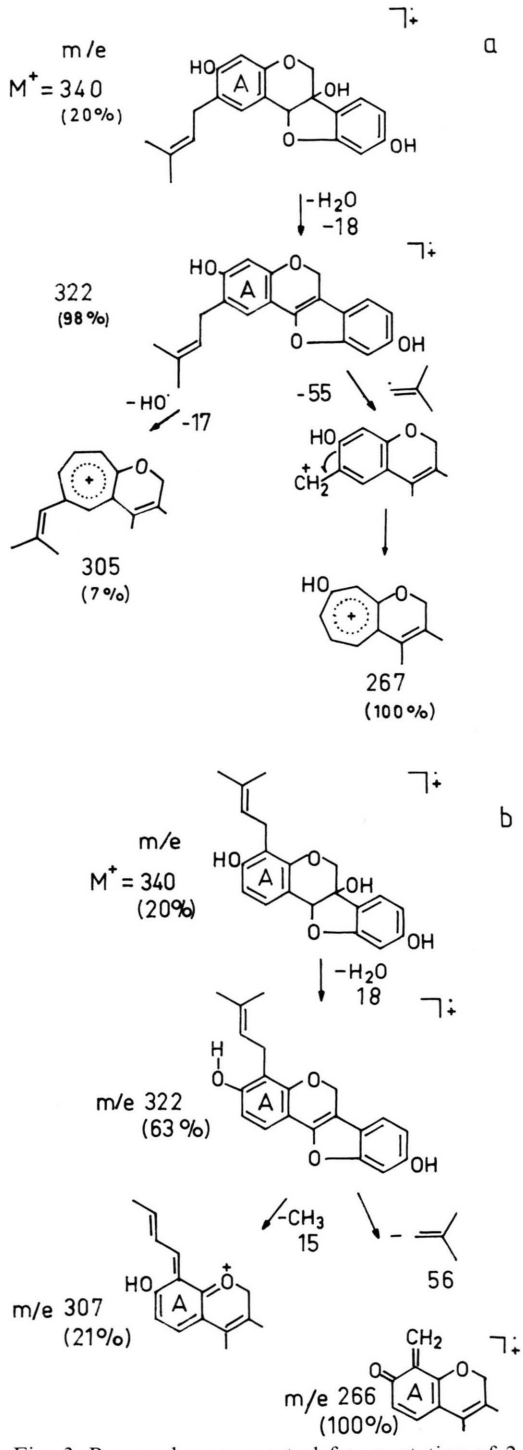

Fig. 3. Proposed mass spectral fragmentation of 2-DMA-THP (a) and 4-DMA-THP (b). Because of the symmetry of the THP-molecule the mass spectrum does not allow a decision whether the DMA-residue is attached to the A- or D-ring.

Unequivocal proof that the minor product is the 4-DMA isomer **2** was obtained by a 250 MHz ^{1}H-NMR spectrum. The chemical shifts and coupling constants of THP and of the two dimethylallyl isomers are listed in Table II. As one would expect for the 4-DMA isomer, the signal for H-4 is missing. A new doublet appears at δ 6.59 ppm which can be assigned to H-2 and which shows an ortho-coupling to H-1. This assignment was confirmed by a decoupling experiment where irradiation at H-2 leads to a broad singlet for the signal of H-1.

Restricted rotation of the DMA substituent in the 4-isomer can be concluded from the large difference in chemical shifts of the two methyl groups (H-15,15′), which both appear as broad doublets with long-range coupling to H-13. In contrast, in the 2-isomer the two methyl groups give only one broad singlet.

Biological activity

The biological activity of THP, 2-DMA-THP and a mixture of glyceollins was compared in a combined TLC-fungitoxicity assay against *Cladiosporium cucumerinum* [17]. From the bioautogram shown in Fig. 4 no inhibition can be seen with THP under these conditions, whereas 2-DMA-THP showed a clear inhibition zone, which was somewhat smaller than that of the glyceollin isomers. In a biotest with the bacterium *Erwinia carotovora* [22], however, clear inhibition zones were obtained with all 3 substances.

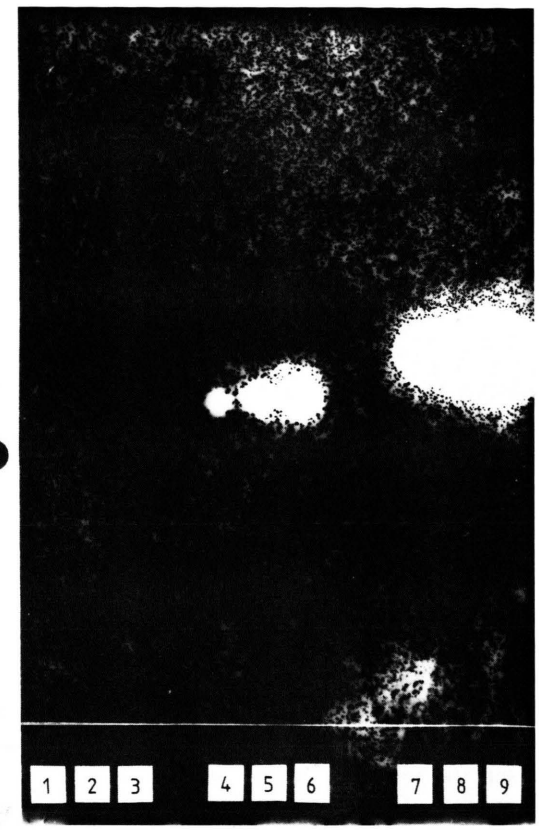

Fig. 4. TLC-fungitoxicity assay with *Cladosporium cucumerinum*. The silica gel plate was developed with solvent system 1. 2,4 and 10 µg of each substance was applied. 1–3 THP; 4–6 2-DMA-THP; 7–9 mixture of glyceollins I–III. The black spot at the left side indicates position of THP on the TLC plate.

Table II. ^{1}H-NMR-Spectra of 3,6a,9-trihydroxypterocarpan (THP) and the dimethylallyl-substituted isomers at 250 MHz in d_6-acetone.

Proton	4-DMA-THP		2-DMA-THP		THP	
	δ [ppm]	J [Hz]	δ [ppm]	J [Hz]	δ [ppm]	J [Hz]
H–1	7.15 d	7.8	7.17 s	–	7.31 d	8.3
H–2	6.59 d	7.9	–	–	6.56 q	8.2/2.4
H–4	–	–	6.35 s	–	6.32 d	2.4
H–6	4.06 d	11.0	3.98 d	11.4	4.02 d	11.5
H–6′	4.19 dd	11.0/1	4.13 dd	11.4/1	4.15 dd	11.3/1
H–7	7.23 d	7.0	7.20 d	8.5	7.20 d	8.3
H–8	6.44 q	7.9/2.2	6.42 q	8.3/2.0	6.42 d	8.2/2.2
H–10	6.24 d	2.2	6.25 d	2.0	6.24 d	2.1
H–11a	5.29 s *	–	5.24 s *	–	5.26 s *	–
H–12	3.30 d *	7.0	3.29 d *	7.1	–	–
H–13	5.20 m	7.8/1.8	5.36 m	7.0/1.7	–	–
H–15	1.58 d *	1.8	1.75 s *	–	–	–
H–15′	1.73 d *	1				
6a–OH	4.49 s	–	4.88 s	–	–	–

* Broad signals

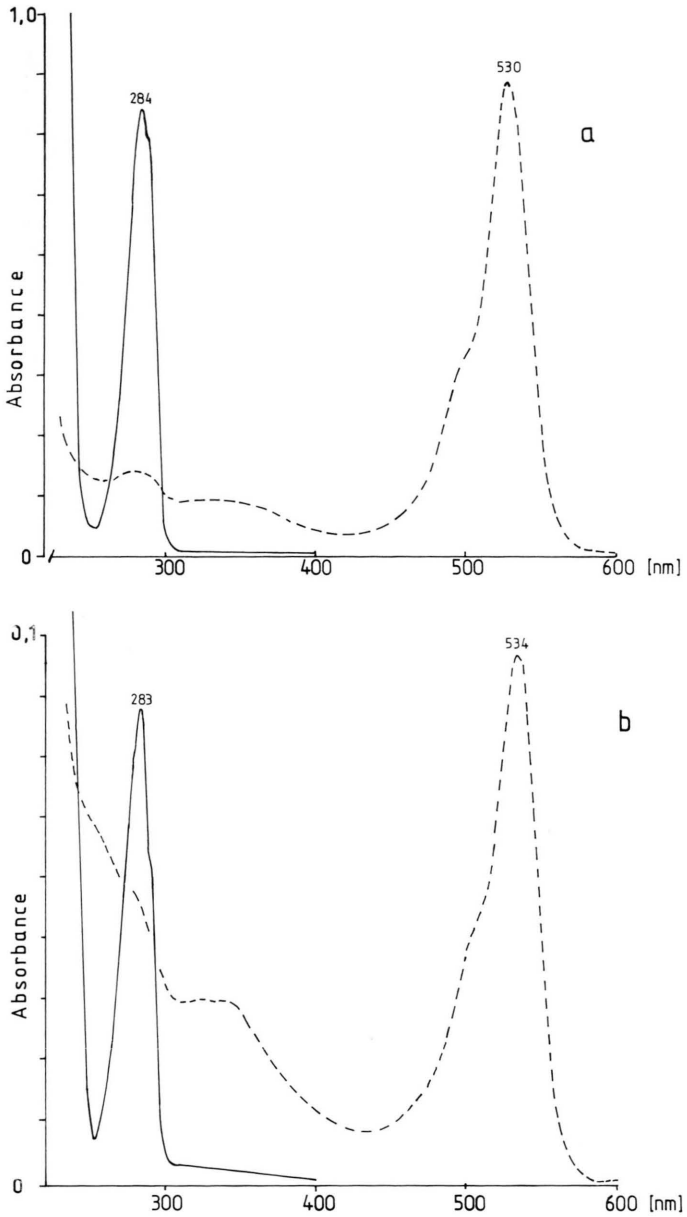

Fig. 5. UV-Spectra of 4-DMA-THP (a) and 2-DMA-THP (b) in ethanol (——) and spectra of red solution after addition of 4 N KOH (- - -).

Red pigment from 3,6a,9-trihydroxypterocarpans and its derivatives

Upon treatment of soybean cotyledons with Pms-elicitor a red colouration appears on the surface which is in contact with the elicitor. It was reported that the intensity of the red colour is roughly proportional to the glyceollin content in the wound droplet [12].

We observed that solutions of 3,6a,9-trihydroxypterocarpan or its derivatives turn red upon addition of KOH in presence of molecular oxygen, whereas the glyceollin isomers and 3,9-dihydroxypterocarpan do not show this reaction. The 6a-hydroxyl group is therefore needed for colour formation, but whether the presence of the 9-OH group is also necessary is not known. Addition of sodium dithionite bleaches the colouration. Fig. 5 shows the spectra of the 4-

and 2-DMA-THP in ethanol and after addition of 4 N KOH. The fine structure of the UV-spectra of the isomers is different; the red colour has an absorbtion maximum at 530 and 534 nm, respectively. The corresponding red colour from THP has a λ_{max} at 514 nm. Addition of HCl shifts the absorption maxima to about 460 nm.

An intense red pigment from 2-DMA-THP and one from THP could be purified by reversed phase HPLC on a LiChrosorb RP-18 column. However, the pigment derived from THP was extremely unstable. The pigment from 2-DMA-THP was obtained in HPLC as a single peak. A mass spectrum of this compound gave a molecular ion at *m/e* 338 (12%) and major fragment ions at *m/e* 323 (30%), 320 (76%), 305 (100%), and 152 (50%). According to the shape of the ion current the spectrum originates from two substances which differ by 18 mass units (M^+-H_2O). The M^+ ion at *m/e* 338 and the fragmentation pattern are consistent with the quinoid structure shown in Fig. 6. Elimination of a methyl radical from the parent compound or from the compound formed after elimination of water leads to the observed major ions at *m/e* 323 and 305, respectively. An analogous elimination of methyl radicals has been observed with ubiquinones [13]. A quinoid structure for the pigment is also in agreement with the bleaching by sodium dithionite [14]. It must be mentioned that the colour reaction is complex and obviously leads to several products, of which only one was isolated. When the red solution resulting from treatment of [^{14}C]-2-DMA-THP with KOH was analysed by TLC in solvent system 1 about 8 radioactive products were found by scanning the plate.

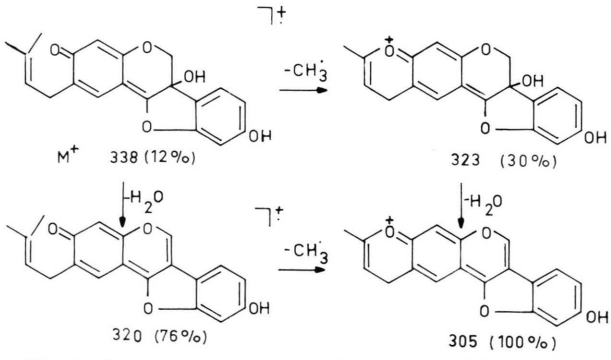

Fig. 6. Proposed structure for the protonated form of one of the pigments from 2-DMA-THP and its mass spectral fragmentation.

Discussion

The identification of two products from the dimethylallyl-transferase reaction as 2- and 4-DMA-THP is consistent with the occurrence of the glyceollins shown in Fig. 1. The finding that 2- and 4-DMA-THP also occur as natural products in elicitor-treated soybean cotyledons and soybean cell cultures strongly corroborates the assumption that these compounds are the first prenylated intermediates in the biosynthesis of glyceollins.

In analogy to the pair phaseollin/phaseollidin [16] we propose the name glyceollidin I and II for 4- and 2-DMA-THP, respectively. Having available the data and the reference substance of our enzymatic product, Ingham *et al.* recently also found 2-DMA-THP in leaves of soybean treated with sodium iodoacetate or *Pseudomonas pisi* [15]. These authors propose the name glyceocarpin for this isomer.

Our results indicate that synthesis of 2-DMA-THP and that of 4-DMA-THP are catalysed by two different enzymes [10]. Under the conditions used the internal pool of the 4-isomer is only 2–3% of that of the 2-isomer, whereas glyceollin I, which corresponds to the 4-isomer, is the major glyceollin which accumulates in cotyledons.

The biotest with *C. cucumerinum* (Fig. 4) shows that introduction of the dimethylallyl substituent into THP increases its fungitoxicity against this organism. According to this result 2-DMA-THP can be classified as a phytoalexin.

We have never observed glyceollin IV, which is the 3-O-methyl derivative of 2-DMA-THP [3], in elicitor-treated cotyledons or cell cultures.

When a correlation between intensity of the red colouration and glyceollin accumulation was found [12], it was not known that the major compound which accumulates in elicitor-treated cotyledons is THP [9, 18]. We have now shown that this red pigment originates from THP or its prenylated derivatives. Glyceollins I–III do not give the red colour with alkali in the presence of molecular oxygen. Formation of the red colour with alkali is therefore a convenient test for the presence of molecules with the 3,6a,9-trihydroxypterocarpan skeleton.

Acknowledgements

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