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IDENTIFICATION OF PLANT GROWTH INHIBITORS PRODUCED BY PHOTOLYSIS OF VIOLAXANTHIN

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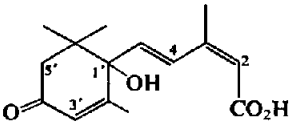
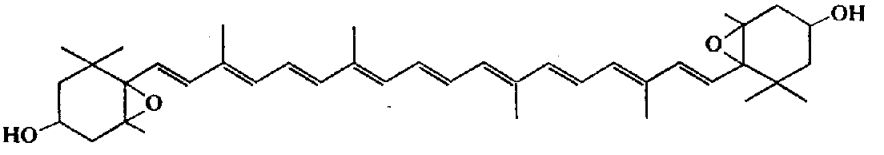
Abstract—Certain plant xanthophylls produce growth inhibitors on illumination. Photo-oxidation of violaxanthin (II) has been shown to yield a neutral inhibitor with a biological activity approaching that of abscisic acid (I). Structure (VI) is proposed for the neutral inhibitor. Two other products of the photo-oxidation have been isolated and characterized as 4-(1′,2′-epoxy-4′-hydroxy-2′,6′,6′-trimethyl-1′-cyclohexyl) *trans*-3-buten-2-one (IV) and 1,3-dihydroxy-3,5,5-trimethylcyclohexylidene-4-acetic acid lactone (V). The latter is shown to be identical with loliolide, isolated from several plant species.

INTRODUCTION

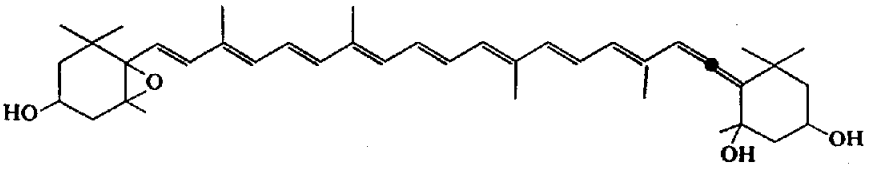
STRUCTURAL similarities between the naturally occurring growth inhibitor, abscisic acid (I), and carotenoid pigments led to the suggestion that these pigments could be precursors of the inhibitor.[1] As the major constituent of inhibitor β is now considered to be abscisic acid (ABA) and plants grown in light contained more inhibitor β than etiolated plants,[2] it was further suggested that light was involved in the conversion. The results of experiments in which inhibitors of cress seed germination were produced by the illumination of extracted nettle leaf carotenoids supported this idea.[1] Whilst all carotenoids were not equally inhibitory after exposure to light, the two xanthophyll epoxides, violaxanthin (II) and neoxanthin (III), were shown to be most effective.[3]



Abscisic acid (I)



Violaxanthin (II)



Neoxanthin (III)

[1] H. F. TAYLOR and T. A. SMITH, *Nature* **215**, 1513 (1967).
[2] S. T. C. WRIGHT, Ph.D. Thesis, University of Bristol (1954).
[3] H. F. TAYLOR, Plant Growth Regulators, *Soc. Chem. Ind. Monograph* No. 31, p. 22 (1968).

The inhibitor produced by the illumination of violaxanthin on filter paper was shown to be non-acidic, although evidence for its conversion to an acidic inhibitor by cress seeds was obtained.[3,4] The formation of plant growth inhibitors by the action of light on certain leaf xanthophylls suggested an explanation for some of the responses of plants to blue light, and evidence in support of this has been presented.[4] For further physiological investigations, however, isolation and identification of the active inhibitor is essential. In this paper we report the isolation and chemical characterization of substances produced by the illumination of violaxanthin. Their growth inhibitory activity is assessed and compared with that of known growth inhibitors.

RESULTS AND DISCUSSION

Orange peel was found to be a very convenient source of violaxanthin and the extraction procedure employed is outlined in the Experimental. The photo-oxidation was effected by adsorbing the pigment onto cellulose powder and exposing an aqueous slurry of this to bright daylight. The products were then obtained by filtration and ether extraction of the aqueous filtrate.

TLC of the ether extract showed the presence of numerous compounds; however, cress seed germination and wheat coleoptile section tests indicated a sharp major zone of inhibition at R_f 0·4 on silica gel plates developed with benzene–ethyl acetate (1:1). Subsequent efforts were directed towards the separation and identification of substances in this region. Column and thin-layer chromatographic techniques eventually led to the isolation of two crystalline compounds, m.p. 59° and 149°, together with a "TLC pure" non-crystalline substance which was highly active in suppressing seed germination and inhibiting coleoptile growth.

The compound, m.p. 59°, gave a yellow coloration with 2,4-dinitrophenylhydrazine, indicating the presence of an aldehyde or ketone function. The mass spectrum gave the mol. wt. as 224 and indicated $C_{13}H_{20}O_3$ as the probable molecular formula. I.r. maxima, in $CHCl_3$, at 3420, 1675 and 1625 cm^{-1} suggested the presence of hydroxyl and $\alpha\beta$-unsaturated carbonyl functions. In ethanol, a single u.v. maximum was present at 233 nm ($\epsilon = 10{,}800$). NMR data are summarized in Table 1. Three methyl groups, having band positions closely

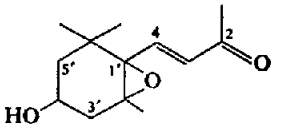
TABLE 1. NMR DATA FOR COMPOUND IV

Bands*	Assignment
2·90, d, 1 H (J = 16 Hz)	Proton at C-3
3·66, d, 1 H (J = 16 Hz)	Proton at C-4
6·04, m, 1 H	Proton at C-4′
7·4–8·7, m, 4 H	Protons at C-3′ and C-5′
7·70, s, 3 H	Methyl at C-1
8·3, s, 1 H	Hydroxyl (disappearing with D_2O)
8·78, s, 6 H	Methyls at C-2′ and C-6′
9·00, s, 3 H	Methyl at C-6′

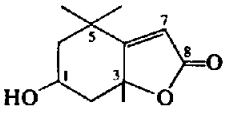
* Spectra were determined at 100 MHz in $CDCl_3$ using TMS as internal standard. Band positions are given in τ values, s = singlet, d = doublet and m = multiplet.

[4] H. F. TAYLOR, Ph.D. Thesis, London University (1969).

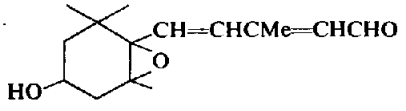
resembling those of the methyl groups in the violaxanthin end rings,[5] are apparent. An additional methyl deshielded by an adjacent carbonyl group and a *trans*-olefinic bond in conjugation with the carbonyl group can also be recognized.



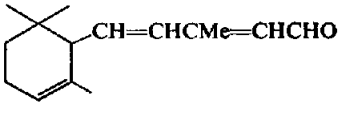
(IV)



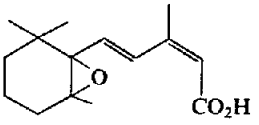
Loliolide (V)



(VI)



(VII)



(VIII)

These data characterize the compound, m.p. 59°, as 4-(1′,2′-epoxy-4′-hydroxy-2′,6′,6′-trimethyl-1′-cyclohexyl)-*trans*-3-buten-2-one (IV). It is evidently formed from violaxanthin by oxidative cleavage of the polyene chain. Bioassay results are given in Fig. 1 and Table 3 and show that while the substance is effective in the tests, it is not a powerful plant growth inhibitor.

TABLE 2. NMR DATA FOR COMPOUND V

Bands*	Assignment
4·26, s, 1 H	Proton at C-7
5·62, m, 1 H	Proton at C-1
7·4–8·6, m, 4 H	Protons at C-2 and C-6
8·18, s, 3 H	Methyl at C-3
8·3, s, 1 H	Hydroxyl (disappearing with D_2O)
8·50, s, 3 H	Methyl at C-5
8·70, s, 3 H	Methyl at C-5

*Footnotes as for Table 1.

The crystalline compound, m.p. 149°, was very similar in chromatographic behaviour to the neutral inhibitor but was easily purified by recrystallization. It had negligible biological activity (Fig. 1 and Table 3) and did not form a 2,4-dinitrophenylhydrazone. In the mass spectrum, the molecular ion appeared at *m/e* 196 suggesting $C_{11}H_{16}O_3$ and a fragment ion involving loss of water was also prominent. The compound had u.v. maximum in ethanol at 215 nm ($\epsilon = 13{,}300$) and i.r. absorption bands in $CHCl_3$ at 3460, 1730 and 1625 cm^{-1}. This is consistent with the presence of hydroxyl and $\alpha\beta$-unsaturated lactone functions. The NMR

[5] L. CHOLNOKY, K. GYORGYFY, A. RONAI, J. SZABOLCS, GY.TOTH, G. GALASKO, A. K. MALLAMS, E. S. WAIGHT and B. C. L. WEEDON, *J. Chem. Soc.* (C) 1256 (1969).

TABLE 3. ASSESSMENT OF INHIBITORY ACTIVITY OF COMPOUNDS IN THE CRESS SEED GERMINATION TEST

Compound	Concentrations (ppm) tested	Concentration (ppm) for inhibition*
(IV)	10–250	25
(V)	10–250	250
Coumarin	10–250	25
Neutral inhibitor (VI)	0·1–10	0·2
(±)ABA	0·1–10	0·2

* Inhibition greater than 50%, assessed after 72 hr.

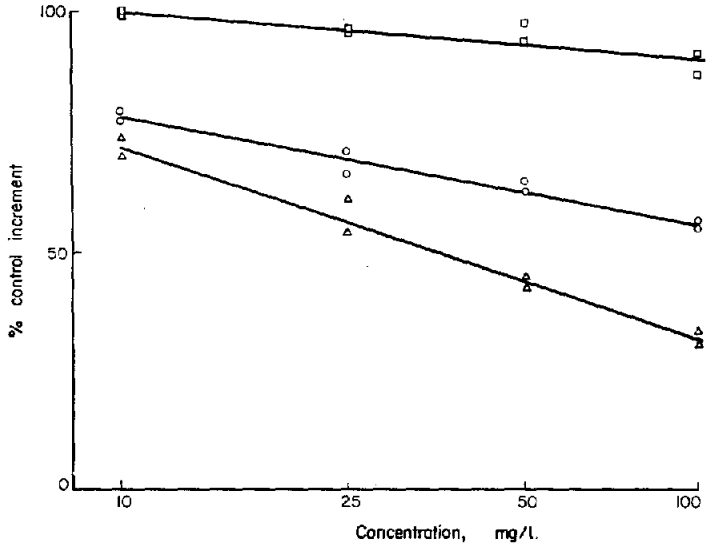


FIG. 1. ASSESSMENT OF INHIBITORY ACTIVITY IN THE WHEAT COLEOPTILE SECTION TEST OF COMPOUND IV (○), COMPOUND V (□) AND COUMARIN (△). THE MEAN INCREMENT OF TEN TEST SECTIONS IS EXPRESSED AS A PERCENTAGE OF THE MEAN INCREMENT OF CONTROL SECTIONS.

spectrum (Table 2) exhibited a sharp singlet in the olefinic region and indicated that oxidative cleavage of the polyene chain, together with double bond migration and epoxide ring opening, had occurred to give (V). The compound is therefore 1,3-dihydroxy-3,5,5-trimethylcyclohexylidene-4-acetic acid lactone. It is thus identical with loliolide, a lactone, m.p. 149°, isolated from *Lolium perenne* (perennial ryegrass) and also from the unrelated *Digitalis lanata* and *Fumaria officinalis*.[6] A detailed m.p. and spectral comparison confirmed this. As violaxanthin is a widespread plant pigment, it seems very likely that it is the biogenetic precursor of loliolide.

The highly active inhibitor was present in a very low concentration and was separated with difficulty from residual loliolide by TLC on cellulose. It was readily detected by spraying with 2,4-dinitrophenylhydrazine reagent with which it gave an orange spot on warming. When chromatographed in several thin-layer systems it remained homogenous. In the u.v.

[6] R. HODGES and A. L. PORTE, *Tetrahedron* **20**, 1463 (1964).

it had an intense maximum at 284 nm. Bioassay data indicated a level of activity in the wheat coleoptile section test (Fig. 2) about half that of (±)ABA, but similar to (±)ABA in the cress seed germination test (Table 3).

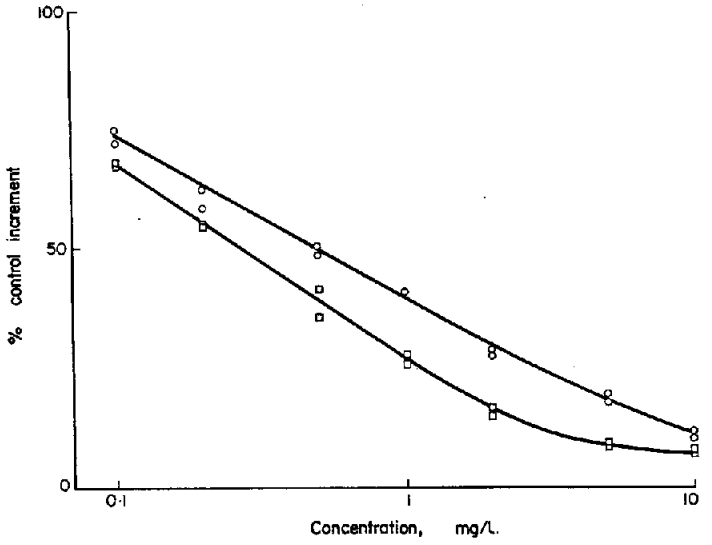


FIG. 2. ASSESSMENT OF INHIBITORY ACTIVITY IN THE WHEAT COLEOPTILE SECTION TEST OF "NEUTRAL INHIBITOR" (○) AND (±)-ABSCISIC ACID (□), EXPRESSED AS IN FIG. 1.

The neutral inhibitor gave an acetate, which had a similar u.v. maximum and the same 2,4-dinitrophenylhydrazine colour reaction; it retained about half the biological activity of the parent compound. The facile formation of an acetate is good evidence that the secondary hydroxyl group of violaxanthin has been retained in the inhibitor. In view of the chromatographic similarity with IV and V, this is likely to be the only hydroxyl present. The u.v. maximum is consistent with the presence of a $\beta\delta$-disubstituted dienal and this leads to the formulation of VI as the probable structure. As a model chromophore, α-ionylidene acetaldehyde (VII) is reported[7] to have λ_{max} 285 nm in ethanol.

It is of interest at this point to refer to the work of Tamura and Nagao.[8] In studies on synthetic analogues of ABA, they found that 5-(1′,2′-epoxy-2′,6′,6′-trimethyl-1′-cyclohexyl)-3-methyl-*cis,trans*-2,4-pentadienoic acid (VIII) and its esters were comparable with (±)ABA in suppressing the growth of rice seedlings. Analogues without the epoxide group had vastly lower activity as did those with a *trans,trans*-pentadienoic acid side-chain.

Structure VI could therefore account for the observed biological activity although, on the basis of known structure–activity relationships, it would be necessary to postulate the presence of a *cis–trans*-isomer. This does not seem unlikely as carotenoids frequently occur as *cis*-isomers and stereomutation could also have occurred in the photochemical stage. In the near future we hope to employ GLC for investigating the presence of isomers and to obtain sufficient pure material to confirm the proposed structure by the usual spectroscopic techniques.

[7] C. D. ROBESON, J. D. CAWLEY, L. WEISLER, M. H. STERN, C. C. EDDINGER and A. J. CHECHAK, *J. Am. Chem. Soc.* **77**, 4111 (1955).
[8] S. TAMURA and M. NAGAO, *Planta* **85**, 209 (1969).

EXPERIMENTAL

Cress Seed Germination Test

Cress seeds (*Lepidium sativum* cv. Curled Green) were sown on moistened filter papers and incubated in a darkened high humidity chamber at 23·5° for 72 hr. The germination of seeds on filter papers treated with growth inhibitor was compared with that on clean papers.

Wheat Coleoptile Section Test

Wheat seeds (*Triticum vulgare* cv. Eclipse) were germinated on moistened filter paper for 70 hr at 23·5° in darkness. Ten sections (10 mm in length) cut from uniform 20 mm coleoptiles were incubated in a closed specimen tube (100 × 13 mm) with a filter paper (90 × 10 mm) and buffer solution[4] (1 ml) on a darkened klinostat. After 18 hr the growth of coleoptile sections incubated with papers previously treated with inhibitor were compared with that of "clean paper" controls.

Extraction of Violaxanthin from Orange Peel

Orange fruits (10 kg) were peeled with a vegetable peeler and the pith-free rind (1·5 kg) minced and then macerated (M.S.E. Atomix) with methanol (5 l.). The macerate was filtered and the filtrate used for the maceration of a further 1·5 kg of rind. The combined pulp was extracted overnight with ether and re-extracted twice with ether (8 l. total vol.). On concentration at reduced pressure, the extract separated to give an upper intensely pigmented layer. The lower layer was extracted with light petroleum and the extract combined with the pigment layer. After partitioning with 90% aq. methanol, the light petroleum solution was concentrated, benzene added, and again reduced to a small volume.

Ethanol (100 ml) and 60% aq. KOH (10 ml) were added to the pigment and saponification carried out by heating for 5 min in a darkened flask on a steam bath in N_2. After cooling, the pigment solution was diluted with water (3 vol.) and extracted three times with ether (1 vol.). The ether solution was washed, dried, concentrated and redissolved in benzene (200 ml).

Violaxanthin was separated from other carotenoid pigments by chromatography on basic $ZnCO_3$. A column was packed dry in a sintered-glass funnel (105 mm dia.) with a layer of sand on the sintered glass and on the filter paper placed on the $ZnCO_3$ (130 g). After irrigation with benzene, the pigment solution was added and separation obtained by development with benzene. The violaxanthin band, which migrated slowly, was excavated and eluted with ether (500 ml).

Illumination of Violaxanthin

Violaxanthin (150 mg, estimated spectrophotometrically) in ether (500 ml) was adsorbed onto cellulose powder (100 g) and the ether allowed to evaporate. It was then dispersed in distilled water (1 l.) and placed in five white enamelled trays (30 × 30 cm). These were illuminated for 6–7 hr in bright daylight when photobleaching to a pale yellow occurred. The cellulose was filtered and washed with distilled water. Sodium sulphate was added to the filtrate which was then ether extracted. The extracts were dried and evaporated.

Separation of Products of Violaxanthin Illumination

The crude material (136 mg) obtained was dissolved in benzene–EtOAc (1 ml, 2:1, v/v) and chromatographed on a column of silicic acid (30 g) using the same solvent mixture. Five-ml fractions were collected and examined on TLC (Merck silica gel G, benzene–EtOAc, 1:1, v/v) and for growth inhibition in the wheat coleoptile section test.

Fractions 48–59 were found to have high inhibitory activity, 0·2 ml of each yielding sufficient inhibitor to reduce coleoptile section growth by over 50 per cent. These fractions contained a component, R_f *ca.* 0·4, which gave a strong orange spot on spraying with 2,4-dinitrophenylhydrazine reagent and warming. They were bulked and evaporated to yield the "crude inhibitor".

Fractions 64–74 contained a component which gave a yellow colour with 2,4-dinitrophenylhydrazine and had an R_f slightly lower than that of the inhibitor. It was purified by PLC (Merck silica gel GF_{254}) and crystallized from $CHCl_3$–light petroleum to yield 4-(1′,2′-*epoxy*-4′-*hydroxy*-2′,6′,6′-*trimethyl*-1′-*cyclohexyl*)-*trans*-3-*buten*-2-*one* (IV) as colourless prisms (4 mg), m.p. 59°.

The "crude inhibitor" was further purified by PLC (Merck silica gel GF_{254}) and was detected as a quenching zone which corresponded with an orange 2,4-dinitrophenylhydrazine reaction on spraying the edges of the plate. It was thus obtained as a pale yellow gum (6·5 mg) which was taken up in a little ether. The solution was kept at 0° when tiny crystals formed. The ethereal mother liquor was decanted, the crystals washed with cold ether and the washings added to the mother liquors. Recrystallization from CCl_4 gave 1,3-dihydroxy-3,5,5-trimethylcyclohexylidene-4-acetic acid lactone (V) as colourless prisms (3 mg), m.p. 149°.

Evaporation of the mother liquors gave a clear gum (2·5 mg), max 284 and 215 nm. The residual loliolide was removed by PLC on cellulose (Merck cellulose F) using wet hexane for development. Under these conditions, loliolide remained near the origin and the inhibitor migrated as a broad irregular band. The "purified inhibitor" was thus obtained as a clear syrup (1·2 mg), TLC pure and with λ_{max} 284 nm.

Acetylation of Purified Inhibitor

Purified inhibitor (1 mg) was dissolved in pyridine (6 drops) and acetic anhydride (4 drops) was added. The mixture was left at room temp for 24 hr after which it was adsorbed on neutral alumina and eluted with benzene containing 10% EtOAc. The acetylated inhibitor was detected in the eluate by TLC (silica gel G, benzene–ethyl acetate, 1:1) and gave an orange spot at R_f 0·7 with 2,4-dinitrophenylhydrazine reagent. It was further purified by PLC and was obtained as a clear gum, λ_{max} 283 nm.

Acknowledgements—We acknowledge the assistance of Dr. Millard, London School of Pharmacy (for mass spectrometry); Dr. D. Shaw, Varian Associates Ltd. (NMR); and Professor J. W. Cornforth, F. R. S., Shell Research Ltd., for (±)ABA. Our thanks are also expressed to Mr. P. Cozens and Mr. R. P. Townsend for their expert technical assistance, and to Professor R. L. Wain, F.R.S., for his advice and encouragement in these investigations.