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# ABNORMAL METABOLITES PRODUCED BY *DAUCUS CAROTA* ROOTS STORED UNDER CONDITIONS OF STRESS

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**Key Word Index**—*Daucus carota*, Umbelliferae, carrot, ethylene, dihydroisocoumarins, chromones, 6-methoxymellein, eugenin

**Abstract**—The formation of dihydroisocoumarins (I and II), the chromones (III and IV) and scopoletin (V) is induced in carrot roots by storage in the presence of low concentrations of ethylene and by inoculation with various fungi There has been concern over possible toxic effects of dihydroisocoumarins and some preliminary toxicological data on (I), (II) and (IV) are included

## INTRODUCTION

6-METHOXYMELLEIN (II) was first isolated by Sondheimer[1] and shown to be a compound responsible for the bitter taste produced in carrots kept in a commercial cold store for 6–8 weeks at 0° It was later reported[2] that formation of 6-methoxymellein was induced in carrot root tissue inoculated with the fungus *Ceratocystis fimbriata* and that this compound played a part in the resistance of the carrot to fungal attack since it was produced in sufficient amount to inhibit fungal growth [3] Bitterness found in carrots stored in the presence of apples[4] prompted an investigation into the effect of ethylene on carrot roots and it was discovered that ethylene had a catalytic effect on the production of 6-methoxymellein [5,6]

It has subsequently been shown by various workers that the formation of 6-methoxymellein in carrot root tissue can be induced by chemical treatments including mercuric chloride, cupric chloride,[7] indoleacetic acid, 2,4-dichlorophenoxyacetic acid and 2,4,5-trichlorophenoxyacetic acid[6] and by fungal inoculation with a variety of species including *Ceratocystis fimbriata*, *C ulmi*, *Helminthosporium carbonum*, *Fusarium oxysporum* f *lycopersici*,[8] *Thielaviopsis basicola*[9] and *Rhizopus stolonifer* [10]

It is clear that a wide range of stresses will induce carrot tissue to produce abnormal metabolites and we have carried out a more detailed investigation to see whether other compounds related to 6-methoxymellein are produced Ethylene is a very convenient agent for producing stress and we have found that fresh carrot roots stored in air containing a very low concentration of ethylene produce a range of abnormal phenolic metabolites which are not produced in detectable amounts in control samples

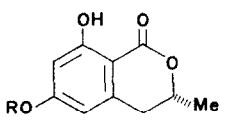
[1] SONDHEIMER, E (1957) *J Am Chem Soc* **79**, 5036
[2] CONDON, P and KÚC J (1960) *Phytopathol* **50**, 267
[3] HERNDON, B A , KÚC, J and WILLIAMS, E B (1966) *Phytopathol* **56**, 187
[4] CAROLUS, R L and ELLS, J E (1959) *Am Veg Grower* **7**, 38
[5] CARLTON, B C , PETERSON, C E and TOLBERT, N E (1961) *Plant Physiol* **36**, 550
[6] CHALUTZ, E , DEVAY, J E and MAXIE, E C (1969) *Plant Physiol* **44**, 235
[7] KÚC, J (1972) in *Phytotoxins in Plant Diseases* (WOOD, R K S , ed ), p 89, Academic Press, New York, and in the case of mercuric chloride supported by our own experiments
[8] CONDON, P , KÚC, J and DRAUDT, H N (1963) *Phytopathol* **53**, 1244
[9] HAMPTON, R E (1962) *Phytopathol* **52**, 413
[10] MENKE, G H , PATEL, P N and WALKER, J C (1964) *Z Pfl Krankh* **71**, 128

## RESULTS AND DISCUSSION

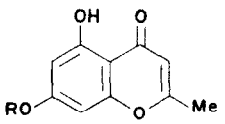
Preliminary small scale experiments using five varieties of carrot (Nantes, Royal Chantenay, Berlicum, Autumn King and White Belgian) showed that 6-methoxymellein (II), and at least two other phenolic compounds, not normally detected in controls, were present in the $Et_2O$ extract of carrot roots stored in air containing 5–100 ppm of ethylene for 5–7 days at 15° and 95% relative humidity Large batches of carrots were subsequently exposed to ethylene under controlled conditions and the $Et_2O$ extract from these was chromatographed over silica gel

Initial fractions from column chromatography contained β-carotene followed by a major phenolic fraction which provided colourless plates, m p 75–6°, identified as (−)-6-methoxymellein from spectral data and by comparison with an authentic sample [11] A second major phenolic constituent, $C_{11}H_{10}O_4$, m p 118°, was obtained following further chromatography, and identified from spectral data as eugenin[12] (5-hydroxy-7-methoxy-2-methylchromone) (IV) which has not previously been isolated from carrot but has been isolated from *Eugenia aromatica* [12] Characterization was confirmed by a comparison with synthetic eugenin prepared from phloracetophenone by a Kostanecki–Robinson reaction to give 5,7-dihydroxy-2-methylchromone[13] (III) which was monomethylated with diazomethane

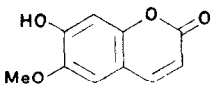
A more polar phenolic, $C_{10}H_{10}O_4$, m p 211°, with similar spectral properties to 6-methoxymellein was identified as (−)-6-hydroxymellein [(−)-6,8-dihydroxy-3-methyl-3,4-dihydroisocoumarin] (I) by direct comparison with an authentic sample obtained from a mutant of *Aspergillus terreus*[14] and with a semi-synthetic sample prepared by demethylation of (−)-6-methoxymellein with hydrogen bromide in acetic acid



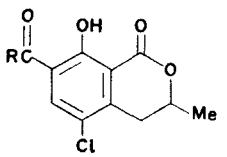

(I) R = H
(II) R = Me



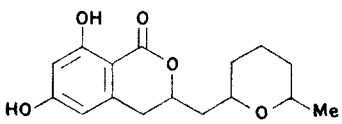

(III) R = H
(IV) R = Me




(V)




(VI) R = NH–CH(COOH)$CH_2C_6H_5$
(VII) R = OH




(VIII)

Small quantities of two other phenolic compounds of similar polarity to (I) were separated by preparative TLC One of these has been tentatively identified as 5,7-dihydroxy-2-methylchromone (III) by UV and TLC comparison with the synthetic compound The other minor constituent gave a highly characteristic brilliant pale blue fluorescence under UV illumination at 254 nm and the characteristic absorption spectrum of a coumarin It was identified as scopoletin (V), by direct TLC, IR and MS comparison with an authentic sample

[11] Kindly provided by Professor J Kuc
[12] Meijer, Th M and Schmid, H (1948) *Helv Chim Acta* **31**, 1603
[13] Gulati, R C, Seth, S R and Venkataraman, K (1934) *J Chem Soc* 1765
[14] Curtis, R F, Harries, P C, Hassall, C H, Levi, J D and Phillips, D M (1966) *J Chem Soc* C, 168

6-Methoxymellein was the major $Et_2O$ soluble phenolic metabolite produced by carrots stored in the presence of ethylene and together with eugenin accounted for >95% of the phenolic material in the extract Much larger quantities of all the phenolic compounds were obtained by using 2 cm thick discs cut from carrot roots, a technique which has been extensively used in studies of fungal inhibition, rather than using whole roots.

Carrot root discs inoculated with *Ceratocystis fimbriata* produced 6-methoxymellein (TLC, UV), eugenin (TLC, UV), 6-hydroxymellein (TLC) and small amounts of 5,7-dihydroxy-2-methylchromone, but the presence of the latter was difficult to establish unambiguously by TLC of the crude extract. TLC examination of the extracts from inoculated carrots indicated that the common carrot storage pathogens *Chalaropsis thielavioides*, *Sclerotinia sclerotiorum*, *Centrospora acerina* and *Botrytis cinerea* induced carrot roots to produce the same range of compounds

The presence of 6-methoxymellein in an $Et_2O$ extract of apparently healthy carrots purchased from a market has been reported [15] In our experiments freshly harvested carrots or carrots stored under control conditions for up to 14 days contained no detectable 6-methoxymellein whereas in old and apparently poorly stored carrots quite large quantities were found. An approximate estimation of the dihydroisocoumarin content of market carrots by UV spectrophotometry showed levels of 6-methoxymellein in the range 10–130 $\mu$g/g fresh weight for 11 samples obtained randomly from a local market during the storage period from January to April 1972 A variety of factors might be responsible for the induction of dihydroisocoumarins in commercial supplies of carrots. Ethylene concentrations >0·3 ppm are found in water-logged soils[16] and might be sufficient to induce 6-methoxymellein formation[6] in carrots stored in field clamps Apart from low concentrations of ethylene in storage atmospheres, surface fungal infection on stored carrots would also be expected to induce dihydroisocoumarin production

In addition to our interests in these compounds in relation to plant pathology we are especially concerned with the possibility that abnormal metabolites produced by plants in response to stress may have undesirable properties not only associated with their flavour but also their potential mammalian toxicity. Undesirable flavour changes were the starting point for the studies in which Sondheimer showed a circumstantial correlation between bitterness in carrots stressed by cold and their content of 6-methoxymellein,[17,18] although this was not the only compound responsible Levels of up to 500 $\mu$g/g were present in such material and levels of approximately 80 $\mu$g/g were found in typically bitter carrots No threshold values for bitterness were however determined either in water or carrot

The possibility of the production of potentially toxic metabolites by plant material under stress has been re-emphasized by recent investigations of abnormal furanoterpenoid metabolites with toxic properties produced by sweet potatoes infected with *Ceratocystis fimbriata* [19] Similar compounds are obtained with mercuric chloride and various other metabolic inhibitors[20] and also after insect attack [21]

The suggestion has been made that all dihydroisocoumarins are toxicologically suspect

[15] Bentley, R K , Bhattacharjee, D , Jones, E R H and Thaller, V (1969) *J Chem Soc* C, 685
[16] Dowdell, R J , Smith, K A , Crees, R and Restall, S W F (1972) *Soil Biol Biochem* **4**, 325
[17] Sondheimer, E , Phillips, W F and Atkin, J D (1955) *Food Res* **20**, 659
[18] Sondheimer, E (1957) *Food Res* **22**, 296
[19] Boyd, M R and Wilson, B J (1972) *J Agr Food Chem* **20**, 428, and references cited therein
[20] Uritani, I , Uritani, M and Yamada, H (1960) *Phytopathol* **50**, 30
[21] Akazawa, T , Uritani, I and Kubota, H (1960) *Arch Biochem Biophys* **88**, 150

and should be evaluated for deleterious effects [22] The ochratoxins[23 24] are highly toxic and the dihydroisocoumarin (VII) derived from ochratoxin-*A* (VI) has been shown to inhibit ADP-stimulated respiration when added to rat liver mitochondria [25] Fungitoxic activity for 6-methoxymellein has been reported[3 26] but no toxicities for higher organisms have been recorded

In the light of these observations some preliminary studies on the acute toxicity of 6-methoxymellein have been carried out The $ED_{50}$ values for the toxicities of 6-hydroxymellein, 6-methoxymellein and eugenin were assayed using the brine shrimp (*Artemia salina*) which has been used for general toxicity studies and more recently for the assay of mycotoxins,[27] the values obtained were 128, 90 and 74 $\mu$g/ml, respectively, which by analogy with other acutely toxic compounds did not indicate high toxicity

In acute toxicity tests in female weanling rats dosed intravenously at levels up to 100 mg/kg with 6-methoxymellein and eugenin, all the rats survived and there were no abnormalities seen at autopsy 2 weeks later Pairs of rats dosed orally at 200, 400 and 800 mg/kg with 6-methoxymellein all appeared normal after 48 hr Recent work on ochratoxin-*A* (VI) in chicken,[28] rat[29] and trout,[30] reported whilst our studies were in progress, indicates that the acute toxicity of this compound is not associated with the dihydroisocoumarin moiety of the molecule but is a property of intact ochratoxin-*A* toxicity is lost on hydrolysis to the corresponding dihydroisocoumarin (VII) [31]

Sasaki *et al* [32] have reported that mellein and 4-hydroxymellein have an $LD_{50}$ of 250–500 and 1000–1500 mg/kg, respectively, in mice The $ED_{50}$ of cladosporin[33] (VIII) against brine shrimp is 54 $\mu$g/ml which also indicates a low toxicity It is not surprising therefore that 6-methoxymellein has a low acute toxicity towards both the brine shrimp and the rat These results indicate that concern over the toxicity of dihydroisocoumarins may not be justified

## EXPERIMENTAL

M ps are uncorrected UV spectra were determined in EtOH Relative intensities of 'top ten' MS peaks are given in parenthesis NMR spectra were recorded on a Varian HA-100 or XL-100 spectrometer using tetramethylsilane as internal standard

Carrots (*Daucus carota* varieties Nantes, Royal Chantenay, Berlicum, Autumn King and White Belgian) used in preliminary experiments were grown at this Institute and were harvested immediately before use, as required, during September–November Royal Chantenay carrots for large scale preparative experiments were obtained locally when required Whole roots (washed and air dried) were placed in chambers with 95% RH at 15° and a stream of $CO_2$-free air or $CO_2$-free air containing a low concn ethylene (5–100 ppm) was passed over them at a constant rate for a period of 5–7 days The minced carrot root tissue from ethylene exposed or control samples was stored in jars covered by redistilled peroxide free $Et_2O$ for 1 week at 1 The $Et_2O$ extract was decanted and the residue washed with more $Et_2O$ The combined extracts were evaporated and examined qualitatively by TLC on silica in the solvent systems (1) $C_6H_6$–MeOH–HOAc (10 2 1) and (2) $CHCl_3$ Developed plates were visualized by spraying with diazotized *o*-dianisidine (0 5% solution in

[22] LILLEHOJ, E B , CIEGLER, A and DETROY, R W (1970) in *Essays in Toxicology*, Vol 2, p 31, Academic Press, New York
[23] VAN DER MERWE, K J , STEYN, P S , FOURIE, L , SCOTT, DE B and THERON, J J (1965) *Nature* **205,** 1112
[24] VAN DER MERWE, K J , STEYN, P S and FOURIE, L (1965) *J Chem Soc* 7083
[25] MOORE, J H and TRUELOVE, B (1970) *Science* **168,** 1102
[26] MCGAHREN, W J and MITSCHER, L A (1968) *J Org Chem* **33,** 1577
[27] HARWIG, J and SCOTT, P M (1971) *Appl Microbiol* **21,** 1011
[28] CHU, F S and CHANG, C C (1971) *J Ass Off Analyt Chem* **54,** 1032
[29] STILL, P E , MACKLIN, A W , RIBELIN, W E and SMALLEY, E B (1971) *Nature* **234,** 563
[30] DOSTER, R C , SINNHUBER, R O and WALES, J H (1972) *Food Cosmet Toxicol* **10,** 85
[31] DOSTER, R C and SINNHUBER, R O (1972) *Food Cosmet Toxicol* **10,** 389
[32] SASAKI, M , KANEKO, Y , OSHITA, K , TAKAMATSU, H , ASAO, Y and YOKOTSUKA, T (1970) *Agr Biol Chem* **34,** 1296
[33] SCOTT, P M , VAN WELBEEK, W and MACLEAN, W M (1971) *J Antibiot* **24,** 747

50% aq MeOH) followed by $NH_3$ solution (0 880 $NH_4OH$–MeOH, 1 1) which gave permanent purple-red spots $R_f$ for the compounds (I–V) in the solvent systems (1) and (2) were (I) $R_f^1$ 0 46, $R_f^2$ 0 12, (II) $R_f^1$ 0 69, $R_f^2$ 0 60, (III) $R_f^1$ 0 44, $R_f^2$ 0 07, (IV) $R_f^1$ 0 63, $R_f^2$ 0 43, (V) $R_f^1$ 0 40, $R_f^2$ 0 19 Scopoletin gave only a feeble reaction with the spray reagent

Samples of carrot tissue for quantitative analysis were macerated in the calculated quantity of absolute EtOH to give an 80% ethanolic solution The resultant slurry was boiled for 10 min , cooled and then filtered to remove extracted carrot tissue and precipitated carotene The filtrate was evaporated and the residue was partitioned between equal volumes of $H_2O$ and hexane ($C_6H_6$-free and redistilled) The aqueous phase was extracted twice more with hexane and the combined hexane extract was dried ($Na_2SO_4$) and evaporated The residue was made up to a standard vol in hexane and the concentration of dihydroisocoumarin was estimated by measuring the absorbance at 265 nm

*Isolation of metabolites* Whole carrot roots (17 5 kg) after exposure to 30 ppm of ethylene in air for 7 days were minced and extracted with $Et_2O$ by the method described above The crude extract obtained (7 29 g) was chromatographed over silica gel (1 5 kg) in $CHCl_3$ The column was eluted successively with $CHCl_3$ and $CHCl_3$ containing increasing proportions of MeOH Early fractions containing β-carotene were discarded Intermediate fractions gave (−)-6-methoxymellein (II) (1 12 g), as colourless plates (from $Et_2O$) m p 75–6°, $[\alpha]_D^{27}$ −52° (*c* 1 23, MeOH), $\lambda_{max}$ 302, 267, 217 nm (log ε 3 74, 4 15, 4 31), $\nu_{max}$ (KBr) 1664, 1636, 1586 $cm^{-1}$ NMR τ 8 50 (3H, *d*, *J* 6 5 Hz), τ 7 14 (2H, *d*, *J* 7 Hz), τ 6 18 (3H, *s*), τ 5 33 (1H, sextet), τ 3 74 (1H, *d*, *J* 2 5 Hz), τ 3 62 (1H, *d*, *J* 2 5Hz), τ −1 22 (1H, *s*, –OH) MS *m/e* 208 ($M^+$, 91), 190 (15), 179 (9), 165 (20), 164 (100), 78 (11), 69 (12), 51 (11), 43 (11), 39 (15) and sitosterol, m p 136° (from $Et_2O$), MW 414 (MS), $[\alpha]_D^{27}$ −32° (*c* 2 52, $CHCl_3$) $\nu_{max}$ (KBr) 1060 $cm^{-1}$, which were separated by column chromatography on Sephadex LH20 eluting with $CHCl_3$–MeOH–petrol b p 60–80° (1 1 1)

Phenolic compounds in later fractions were separated from other fatty material using the same Sephadex LH20 column and solvent system Eugenin (IV) (97 mg) was obtained as pale yellow irregular hexagonal plates m p 118°, $\lambda_{max}$ 290, 256, 249, 230 nm (log ε 3 88, 4 30, 4 30, 4 23), $\nu_{max}$ (KBr) 1666, 1627, 1590 $cm^{-1}$ NMR τ 7 66 (3H, *s*), τ 6 15 (3H, s), τ 3 96 (1H, *s*), τ 3 64 (2H, *s*), τ −2 67 (1H, *s*, –OH) MS, *m/e* 206 ($M^+$, 100), 205 (10), 177 (53), 176 (13), 163 (9), 148 (9), 95 (9), 69 (14), 43 (13), 39 (12) Isolation of the minor phenolic constituents of this extract was difficult due to large quantities of other non-phenolic material An ether extract from sliced carrot roots (1 kg of 2 cm thick discs) exposed to 7 ppm of ethylene in air for 6 days contained relatively larger amounts of the induced metabolites and on chromatography provided (−)-6-methoxymellein (500 mg), eugenin (18 mg) and (−)-6-hydroxymellein (I) (10 mg), microcrystalline m p 211°, $[\alpha]_D^{26}$ −54° (*c* 1 06, EtOH), $\lambda_{max}$ 305, 269, 217 nm (log ε 3 78, 4 10, 4 21), $\nu_{max}$ (KBr) 3420, 3220, 1664, 1635, 1598 $cm^{-1}$ NMR τ 8 47 (3H, *d*, *J* 6 5 Hz), τ 7 12 (2H, *d*, *J* 7Hz), τ 5 29 (1H, sextet), τ 3 76 (1H, *d*, *J* 2 5 Hz), τ 3 65 (1H, *d*, *J* 2 5 Hz) τ −1 19 (1H, *s*, –OH) MS *m/e* 194 ($M^+$, 90), 176 (16), 165 (11), 151 (17), 150 (100), 122 (11), 69 (20), 65 (9), 43 (9), 39 (10)

Further TLC provided a very small quantity of a compound tentatively identified as 5,7-dihydroxy-2-methylchromone (III) Its UV spectrum showed $\lambda_{max}$ 291, 256, 249, 228 nm and TLC comparison with the synthetic compound in several solvents supported its identity A further very minor constituent gave a strong blue fluorescence in UV light at 254 nm and a characteristic UV spectrum $\lambda_{max}$ 341, 290, 256 nm TLC, IR and MS comparison with an authentic sample of scopoletin confirmed the identity of this compound MS *m/e* 192 ($M^+$, 100), 177 (60), 164 (19), 149 (51), 121 (19), 79 (13), 69 (49)

*Fungal inoculation of carrot roots* Carrot root discs (2 cm thick) were inoculated with a spore suspension of *Ceratocystis fimbriata* Agar strips from cultures of the carrot pathogens *Chalaropsis thielavioides*, *Sclerotinia sclerotiorum*, *Centrospora acerina* and *Botrytis cinerea* were inserted into 'V' wounds made in the periderm of whole carrot roots Inoculated and control samples were incubated at 25° for 1–2 weeks The carrot tissue (after removal of agar strips) was extracted with boiling 80% EtOH After removal of the EtOH by evaporation the aqueous residues were extracted with $Et_2O$ The $Et_2O$ extracts were examined by TLC on silica against the reference compounds (I), (II) and (IV) The extract from *C fimbriata* inoculated carrots was separated by preparative TLC ($CHCl_3$ as eluent) and the bands corresponding to 6-methoxymellein and eugenin were removed and their identity was confirmed by measuring UV spectra on the eluted material from each band

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