Conjugated Triene Oxidation Products of α-Farnesene Induce Symptoms of Superficial Scald on Stored Apples

Daryl D. Rowan,*,† Martin B. Hunt,† Simon Fielder,† June Norris,† and Michael S. Sherburn‡

Horticulture and Food Research Institute of New Zealand Ltd., Private Bag 11030, Palmerston North, New Zealand, and School of Chemistry, University of Sydney, Sydney, NSW 2006, Australia

The sesquiterpene α-farnesene (**1**) and its conjugated triene oxidation products accumulate in the skin of apples after harvest and are implicated as the causal agents of superficial scald. Conjugated triene oxidation products and analogues were synthesized and applied to the surface of Granny Smith apples either as vapors or in squalane. Farnesyl hydroperoxide (**2a**), trienol (**2b**), endoperoxide (**3a**), dehydronerolidol (**5**), and cumyl hydroperoxide (**4**) all produced the symptoms of superficial scald when applied at nanomolar doses. Scald-inducing activity was dependent on the mode of application. Farnesyl hydroperoxide (**2a**) was the most active conjugated triene when applied directly to the apple skin. Trienol (**2b**) also induced scald symptoms and partially reversed the inhibition of scald caused by diphenylamine.

Keywords: *Apples; superficial scald; α-farnesene; conjugated triene; autoxidation*

INTRODUCTION

Superficial scald is an important postharvest physiological disorder of apples and pears that appears as a darkening and blackening of the skin after cold storage (*1*, *2*). Browning of the contents of successive layers of hypodermal cells occurs with increasing severity of scald symptoms. In extreme cases of scald, the cell contents of the entire epidermal layer are browned and the sunken appearance of the affected tissue is attributed to the collapse of both the epidermal layer and the outermost cells of the outer cortex (*3*). The occurrence of superficial scald is associated with the oxidation of the sesquiterpene α-farnesene (**1**) (Figure 1) to give conjugated triene oxidation products **2a**, **2b**, **3a**, and **3b**, which accumulate in the apple skin (*4*−*9*). Autoxidation products of α-farnesene, including conjugated triene hydroperoxides or intermediary free radicals (*10*), or 6-methyl-5-hepten-2-one (*11*), have been suggested as the actual causal agents for scald. This is supported by the inhibition of superficial scald by treatment of fruit with antioxidants such as diphenylamine (DPA) (*12*). In vitro autoxidation of α-farnesene (*13*) resulted in the isolation of the conjugated trienol **2b** and the endoperoxide **3b** after reduction of the primary autoxidation products with sodium borohydride. We (*14*), and others (*15*), have recently identified the conjugated triene oxidation products of α-farnesene occurring in stored apples. Surprisingly, the trienol **2b**, rather than the hydroperoxide **2a**, was the major conjugated triene identified. In vitro decomposition of farnesyl hydroperoxide **2a** leads to the endoperoxide **3a** and to 6-methyl-5-hepten-2-one by Hock cleavage (*16*). In vitro autoxidation of **2b** also gave 6-methyl-5-hepten-2-one (*17*).

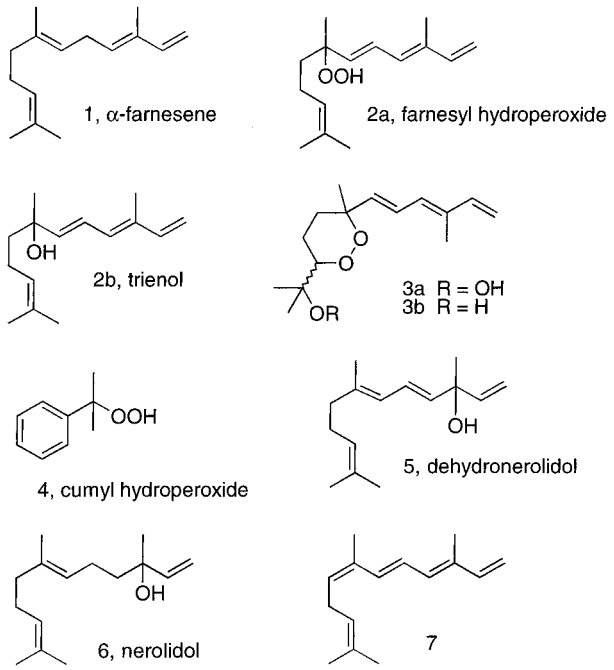

Figure 1. Structures of α-farnesene, conjugated trienes, and conjugated triene analogues.

Natural antioxidants are also present in the apple skin (*6*, *18*−*20*), and hypotheses relating scald to other oxidation processes in the apple skin (*21*−*23*) and to the accumulation of 6-methyl-5-hepten-2-one (*24*) have been presented. Superficial scald has been considered to be a chilling injury (*25*); however, more scald may develop on fruit stored at higher temperatures (10 versus 5 °C) (*26*). Scald and α-farnesene synthesis and oxidation are reduced by treatment of fruit with ethylene inhibitors such as 1-methylcyclopropene and diazacyclopentadiene (*26*−*29*); however, ethylene production is not necessarily correlated with the incidence of scald (*30*). Superficial

* Corresponding author (telephone 64-6-356-8080; fax 64-6-354-6731; e-mail DRowan@hortresearch.co.nz).

† Horticulture and Food Research Institute of New Zealand Ltd.

‡ University of Sydney.

10.1021/jf0015221 CCC: $20.00
Published on Web 05/19/2001

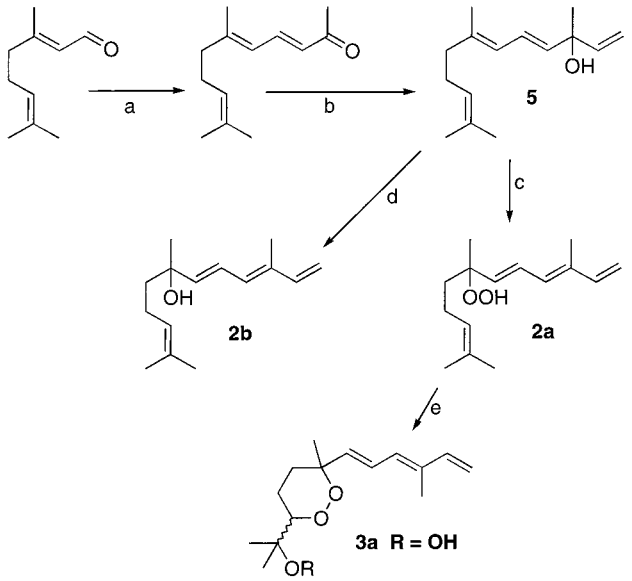

Figure 2. Synthesis of farnesyl hydroperoxide **2a**, trienol **2b**, endoperoxide **3a**, and dehydronerolidol **5** after the method of Fielder et al. (*16*): (a) acetone, $Ba(OH)_2$, reflux, 82%; (b) CH_2=CHMgBr, THF, 71%; (c) H_2O_2, H^+, THF, 100%; (d) H_2O, H^+, THF, 96%; (e) SmI_2, O_2, THF-benzene, 82%.

scald is also reduced by postharvest treatment with antioxidants (*4*, *5*, *31*), by low-oxygen controlled-atmosphere storage (*32*−*34*), by brief anaerobic treatment or exposure to ethanol vapors (*35*−*38*), and by wounding (*19*) or the use of coatings (*39*). The biochemical processes leading to superficial scald are obviously complex and remain largely unknown (*40*).

We have previously identified (*14*) and synthesized the major conjugated triene oxidation products formed during the autoxidation of α-farnesene in the apple skin (*16*, *41*). We wished to test the ability of these compounds to induce the symptoms of superficial scald on apple skin. Previous workers (*4*, *42*) have used ethanol solutions to apply test compounds to apple skin; however, application of solvents results in tissue damage and may also induce scald-like symptoms (*4*). Direct injection into the core cavity (*43*) and vermiculite as an absorbent in polyethylene bags (*35*, *36*) have also been used to test compounds for their ability to affect superficial scald. None of these methods appeared to be suitable in the present case. We needed to develop assay systems that caused minimal injury to the apple skin and which were applicable to the smaller amounts of unstable and reactive materials we had available for study. Here we report the use of such assays to measure the ability of conjugated triene oxidation products of α-farnesene and analogues to induce superficial scald symptoms on stored apples.

MATERIALS AND METHODS

Chemicals. Reagents were obtained from the Aldrich Chemical Co., Inc. (Milwaukee, WI), unless otherwise stated and were used without further purification. Pentane (BDH, HiPerSolv) was distilled through an efficient column before use. Farnesyl hydroperoxide **2a** (6-hydroperoxy-2,6,10-trimethyldodeca-2,7*E*,9,11-tetraene) and trienol **2b** (2,6,10-trimethyldodeca-2,7*E*,9,11-tetraen-6-ol) were obtained by isomerization of dehydronerolidol (**5**), with hydrogen peroxide and water, respectively, as a 73:27 mixture of 9*E*:9*Z* isomers (16) (Figure 2). Dehydronerolidol (**5**) was prepared from geraniol (3,7-dimethyloct-2*E*,6-dienol) by Dess−Martin oxidation to geranial, condensation with acetone to give pseudo-ionone, and subsequent addition of vinylmagnesium bromide according to Figure 2. Endoperoxide **3a** was prepared as a 1.2:1 mixture of stereoisomers by oxidative free radical cyclization of **2a** using samarium(II) iodide in an oxygen atmosphere (*41*). Tetraene (**7**) was prepared as a mixture of isomers by dehydration of **2b** with *p*-toluenesulfonic acid (*16*). All synthetic compounds were fully characterized by ^1H and ^{13}C NMR, IR and UV spectrometry, high-resolution EIMS, and, when appropriate, GC-MS. Freshly synthesized samples of **2a** and **3a** were stored overnight in frozen degassed benzene at −20° C. Before use, the benzene was removed under reduced pressure, the residue dissolved in chilled pentane/ether (9:1) or squalane, and the solution used immediately. Thin-layer chromatography (silica, diethyl ether pentane = 1:5) of pentane and squalane solutions of hydroperoxide **2a** remaining after application to fruit indicated that **2a** remained the major component but that some conversion to **3a** had occurred. Endoperoxide **3a** was present essentially unchanged after application of these solutions to fruit.

Materials. Apples, cv. Granny Smith, Red Delicious, Pacific Rose, and Splendour, were harvested from an orchard in the Hawkes Bay region of New Zealand at the beginning of the commercial harvest period. Granny Smith apples were collected from the middle of the canopy of north-south orientated espalier grown trees in 1997, 1998, and 1999 from one Hawkes Bay orchard at the beginning (April 9−11, 1997; March 30 and April 15, 1998; March 11, 1999) and end (April 23, 1997) of the commercial harvest period. Fruits were collected from the center of the tree and selected for uniform size and color and for the absence of blemishes and sunburn and were stored at room temperature (~20 °C) overnight before use. Before treatment, each fruit was wiped with a soft paper towel to remove debris and spray residues. After treatment, fruits were stored at 1 °C in commercial cardboard apple boxes (ENZA). The handling holes and seams of the boxes were covered with two layers of brown wrapping paper, held in place with adhesive tap, to increase the internal humidity. Diphenylamine-treated fruits were prepared by immersion of wiped fruit for 5 min in a 17.7 mM solution of diphenylamine prepared from a commercial concentrate. Fruits were drained for 5 min and dried by standing on paper towels overnight before treatment. Control fruits were immersed in water.

Vapor Diffusion (Cup) Assay. Polyethylene disks (70 mm diameter) were cut from plastic food bags, soaked sequentially in acetone, heptane, and pentane, and dried under vacuum and fitted into the bottom of plastic drinking cups (height = 90 mm, diameter of rim = 60 mm, diameter of base = 43 mm) by rounding them over the neck of a 100 mL Erlenmeyer flask. Test chemicals were applied onto the disks as solutions in pentane (100 μL), which was allowed to evaporate at room temperature. Pentane (100 μL) was added to the cups used to prepare control fruit. Cumene hydroperoxide (80% solution) was applied as a solution in squalane/pentane (1:4) to reduce the vapor pressure of this compound. After the solvent had evaporated from the bottom of the cup, an apple was placed sideways onto the cup and secured in position using masking tape (3M, 25 mm tape width) so as to seal the apple to the rim of the cup. Apples attached to cups (30−40 per treatment) were placed upright into cartons between corrugated cardboard apple trays.

Squalane Coating Assay. Test chemicals (5−40 mg) were mixed with squalane (2,6,10,15,19,23-hexamethyltetracosane containing 0.02% butylated hydroxytoluene, 760 μL). An aliquot was then used to prepare the next in a series of 10-fold dilutions. Test solutions (20 μL) were applied with a syringe over the surface of one side of the fruit. The squalane solution was immediately dispersed over that side of the fruit with a soft artist's brush previously equilibrated with the test mixture. The nontreated side of the fruit was marked with a black felt pen by drawing a semicircle ~1 cm from the position of the stalk. Fruits (30−40) were placed stalk upright on commercial corrugated cardboard apple trays and packed into cartons and stored as above. Control fruits were treated with squalane.

Measurement of α-Farnesene and Conjugated Trienes in Apple Wax. Portions of heptane (10 mL) were added to glass evaporating dishes (90 mm i.d. × 50 mm deep). Individual apples were placed on top of each dish so that part of the side of the apple surface was immersed in the solvent (*44*). After standing for 20 min, the apple was removed and the diameter of the apple surface in contact with the heptane was measured with calipers. The volume of heptane recovered was recorded to allow for evaporation. Ten apples were extracted per treatment. To determine conjugated trienes, the UV spectrum of the wash solution was measured and the triene concentration calculated as $A_{281} - A_{292}$ with $\epsilon_{281-290} = 25000$ (*45*) and expressed as nanomoles per square centimeter of apple surface area. To determine α-farnesene concentrations, interfering compounds were first removed from the apple wash solution by solid phase extraction (SPE). Samples were diluted 1:10 with heptane (HiPerSolv HPLC), and an aliquot (2 mL) was drawn under low vacuum through a silica SPE column (100 mg, Alltech Extract-Clean RC). The concentration of α-farnesene in the eluant was calculated using A_{232} according to the method given in ref *45* and was expressed as nanomoles per square centimeter of apple surface area. UV spectra were recorded on a Cary 1E spectrophotometer.

Assessment of Tissue Damage. Fruits were removed from cold storage, typically after 6 months when slight scald symptoms were evident, and assessed for superficial scald after 7 additional days of storage in the dark at room temperature. Superficial scald damage was scored both on an intensity scale and as a percentage of the treated area affected. Scald intensity in each year was referenced to a photographic standard of scalded fruit and scored on a 6-point scale from 0 (no visible damage) to 1 (slight scald detected) to 5 (extreme scald showing solid areas of blackened and sunken skin). The mean of these scores (mean scald score) was calculated to summarize these data. The percentage area of the fruit affected by scald was estimated visually and converted to a 5-point scale [0 (no scald), 1 (<10% surface area scalded), 2 (11−30% scalded), 3 (31−50% scalded), 4 (>51% scalded)] for statistical analysis. Distributions of scores were analyzed using chi-squared and Fischer's exact test (SAS). Any other surface defects resulting from the various treatments, for example, surface pitting resembling bitter pit and collapse of the surface due to high doses of **4**, were noted but were not included in the scald scores.

Light Microscopy. Thin slices of glutaraldehyde/formaldehyde-fixed apple tissue were post-fixed in 1% osmium tetraoxide, dehydrated in an acetone series, and infiltrated and embedded in Procure 812 epoxy resin. Sections (1 μm), cut from the heat-cured blocks, were mounted onto glass light microscope slides, stained with 0.05% Toluidine Blue, and examined using a Zeiss Axioplan light microscope.

RESULTS AND DISCUSSION

Development of Bioassay Systems. Given that the causal agent of superficial scald is believed to be volatile (*46*), a vapor diffusion assay was developed which required the test chemical to diffuse onto the fruit surface. Apples were placed and sealed onto the tops of plastic cups into the bottom of which a measured quantity of test chemical was placed. The enclosed side of such fruit showed a slightly higher incidence of scald and slightly higher levels of α-farnesene accumulation, which may be due to the higher humidity found within the cup (*47*). α-Farnesene itself was inactive in this system (unpublished results) in accord with a previous study (*4*) in which ethanolic solutions of α-farnesene were applied directly to the apple skin. Use of deuterated α-farnesene (*48*) in this assay demonstrated diffusion of the test compound onto the apple surface. Equilibration of the deuterated and nondeuterated α-farnesenes in the apple wax was ∼50% complete after 25 days and 100% complete after 40 days at 0 °C (unpublished data). Flow-through vapor exposure assays have also been used to treat fruit with relatively volatile fruit compounds such as hexanal (*49*) and 2-nonanone (*50*). These assays appear to be less suitable for use with the significantly less volatile conjugated trienes, although the trienes would be expected to progressively accumulate in the cuticular wax of the apple skin as postulated for the natural causal agents of superficial scald (*24*, *51*).

The second assay system used squalane rather than ethanol (*4*, *35*, *43*) as the carrier to enable direct application of lipophilic compounds to the apple wax. Squalane is widely used as a human skin lubricant and as a carrier of lipid soluble drugs (*52*) and has been reported to have no effect on volatile production by apples (*53*). Application of squalane to the fruit surface caused a slight intensification of the green color about the lenticels after 1 month of storage at 0 °C. After 6 months at 0 °C, collapse of the skin surface about the lenticels was sometimes observed as small (<1 mm diameter) darker green depressions or pits on the fruit surface similar to the observation of Huelin and Coggiola (*4*) for fruit exposed to aqueous ethanol. No other adverse effects were observed.

Biological Activity of Conjugated Trienes. The vapor diffusion and squalane coating bioassays described above were used to test the biological activity of compounds **2a**, **2b**, **3a**, **4**, and **5** (Figure 1) using freshly harvested fruit (April−May) in 1997, 1998, and 1999. Results are reported for Granny Smith apples only, as this cultivar has been most intensively studied, is highly susceptible to scald, and has a uniform skin color, which makes assessment of scald symptoms easier. Similar symptoms of tissue damage (blackened depressed areas of skin) were observed with the varieties Red Delicious, Pacific Rose, and Splendor, but minor levels of scald symptoms were very difficult to assess. As the incidence of superficial scald varies greatly from year to year, active compounds were retested over successive years. Also for this reason, structure−activity comparisons should be made using data from only any one season.

Scald intensity scores for early- and late-harvest Granny Smith apples treated with farnesyl hydroperoxide **2a**, trienol **2b**, endoperoxide **3a**, and cumyl hydroperoxide **4** using the vapor diffusion and squalane coating assays are recorded in Table 1. Cumyl hydroperoxide was included in these experiments as a model hydroperoxide, its volatility being moderated in the diffusion assays by solution in squalane. Despite this, exposure of the fruit to the two highest doses of cumyl (and *tert*-butyl) hydroperoxide caused extensive damage to fruit surfaces, evident after 1 month of storage as dark brown sunken areas and as dark pitted spots in both diffusion and coating treatments. Some tissue damage, evident as black pitting about the lenticels and at the highest doses as brown sunken areas on the skin, was also observed after 1 month for the highest dose of **2b** in the vapor diffusion assay (slight effect only) and the higher doses of **2a**, **2b**, and **3a** in the squalane coating assay. Little further change in the appearance of the fruit skin occurred in the remaining treatments over the next 4 months, indicating that the above effects may be toxic rather than physiological in origin. After 4 months, the most severely affected treatments (the two highest doses of **4** in the diffusion assay and the highest doses of **2b**, **4**, and **5** in the squalane assay) were

Table 1. Effect of Treatment with Farnesyl Hydroperoxide 2a, Endoperoxide 3a, Trienol 2b, and Cumyl Hydroperoxide on Scald Intensity in Granny Smith Apples (1997 Harvest Year) after 6 Months of Storage at 0 °C Using the Vapor Diffusion and Squalane Coating Assays[a]

	early harvest fruit				late harvest fruit			
	vapor diffusion assay		squalane coating assay		vapor diffusion assay		squalane coating assay	
treatment	dose, μmol/cup	mean scald score	dose, μmol/fruit	mean scald score	dose, μmol/cup	mean scald score	dose, μmol/fruit	mean scald score
control	0.0	1.72[a]	0.0	0.23	0.0	2.96[a]	0.0	1.68[a]
	0.0	2.00[a]			0.0	1.28[b]	0.0	1.00[a]
farnesyl hydroperoxide **2a**	6.047	1.56[a]	2.21	3.56*[a]	10.8	2.16[a]	6.92	3.28*[a]
	0.604	3.16*[b]	0.221	0.48[b]	1.08	2.08*[a]	0.692	1.64[b]
	0.060	1.76[a]	0.022	1.00*[b]	0.11	2.28[a]	0.069	0.68[c]
	0.006	1.48[a]	0.002	0.88*[b]				
trienol **2b**	8.74	4.92*[a]	3.59	5.00*[a]	11.3	4.28*[a]	11.4	4.00*[a]
	0.874	4.04*[b]	0.36	3.48*[b]	1.13	3.72*[b]	1.14	3.64*[a]
	0.087	2.44*[c]	0.036	0.75*[c]	0.11	2.12[c]	0.114	0.88[b]
	0.009	2.36*[c]	0.004	0.48[c]				
endoperoxide **3a**	2.774	1.64[abc]	3.17	2.96*[a]	6.09	2.04*[a]	1.54	1.40[a]
	0.277	2.00[ad]	0.317	1.04*[b]	0.61	1.92*[a]	0.154	0.80[a]
	0.028	2.28[b]	0.032	0.72*[bc]	0.06	2.00[a]	0.015	0.72[a]
	0.003	1.28[cd]	0.003	0.35[c]				
cumene hydroperoxide **4**	54.10	2.25*[ab]	2.78	1.36*[a]	21.7	2.52*[a]	4.76	1.29[a]
	5.41	2.88*[a]	0.34	0.72[a]	2.17	2.68*[a]	0.476	0.64[b]
	0.54	2.00[b]	0.040	1.00*[a]	0.22	1.52[b]	0.048	1.4[a]
	0.054	0.84*[c]	0.005	0.84*[a]				

[a] Scores within treatments followed by the same letter are not significantly different at $P < 0.05$ by Fischer's exact test. An asterisk indicates significant differenence from control distribution.

removed from storage and assessed for scald after a further 7 days of storage at room temperature. The remaining treatments were assessed for scald after 6 months of storage and then 7 days of storage in the dark at ambient temperatures, which intensified the typical scald symptoms that had by then developed in storage. Scald scores from all treatments are presented in Table 1.

All of the compounds tested, **2a**, **2b**, **3a**, and **4**, significantly increased levels of scald in a dose-dependent manner when applied to fruit in low nanomolar amounts. The level of activity for each compound was dependent on both the maturity of the fruit at harvest and the mode of application of the test chemical. For late-harvested fruits, widely acknowledged to be more resistant to scald, higher levels of **2a**, **2b**, and **3a** were required to increase the level of scald in the vapor diffusion assay and higher doses of all four compounds were needed in the squalane coating assay. The order of activity of the compounds appeared to be **2b** ≫ **2a** > **4** > **3a** in the diffusion assay and **2a** ≅ **4** > **2b** ≅ **3a** in the coating assay. The diffusion assay would identify **2b** as the most active compound, being more active than the more volatile cumyl hydroperoxide **4**. However, the diffusion assay is sensitive to both the vapor pressure and stability of the test compounds, and the greater activity of **2b** relative to **2a** probably arises from the increased stability and volatility of **2b**, resulting in a larger dose of this compound reaching the fruit surface. Alternatively, **2b**, rather than **2a**, might decompose to further volatile scald-inducing species, which are retained within the cup used in the assay (*17*, *51*). In the squalane coating assay such differences in compound stability and volatility would not be expected to be so important, and here hydroperoxide **2a** appears to be more active than trienol **2b**. The coating assay would assign **2a** and **4** as being of comparable activity (2 and 5 nmol, respectively, for the least active dose), suggesting the importance of the hydroperoxide functionality for scald-inducing activity. However, the lower activity of endoperoxide **3a** and hydrogen peroxide (Figure 3) and the activity of **2b** suggest other structural motifs must also be considered.

The consistent scald-inducing activity of trienol **2b** (Tables 1−3) is surprising given this compound does not contain a hydroperoxide moiety. Trienol **2b** increased scald severity when applied in the vapor diffusion assay at 9 nmol/fruit with the more susceptible early-harvest fruit and at 36 nmol/fruit when applied directly to the fruit surface in the squalane coating assay (Table 1). Natural concentrations of **2b** in apple skin wax are reported as typically 0.5−3 μg/apple but with levels up to 175 μg/fruit (*14*). This equates, for trienol **2b**, to typical values of 1−7 nmol and up to 400 nmol of endogenous **2b** in the apple tissue used in these bioassays. Such concentrations are within the range of activity for exogenous **2b** applied in both assay systems. Natural concentrations of **2a** and **3a** in fruit are reported as typically 3−4% of the values for **2b** (*14*) and can be estimated at not more than 5 nmol and typically <0.1 nmol in the apple halves used in these assays. Application of these compounds at these levels in the squalane coating assay was sufficient to increase scald symptoms for **2a** but not for **3a** in early-season fruit. The involvement of both **2a** and **2b** but not of **3a** in causing superficial scald is suggested.

Levels of α-farnesene and conjugated trienes in the skin of treated fruit were generally not significantly different from those in controls, although some severely affected samples (e.g., highest doses of **2a** and **4** in the vapor diffusion assay) were observed to have reduced levels of these compounds. Excision and separate analysis of severely damaged areas of apple skin showed that the damaged tissue contained reduced levels of conjugated trienes and α-farnesene. The inclusion of these sections of skin in the analysis may explain the general absence of correlations between α-farnesene, conjugated trienes, and superficial scald levels in these assays.

Table 2. Intensity of Superficial Scald (Mean Scald Score) Produced on Granny Smith Apples Treated with Diphenylamine (DPA) and with Farnesyl Hydroperoxide 2a, Conjugated Trienol 2b, and Cumyl Hydroperoxide 4 (1999 Harvest Year only) in the Vapor Diffusion Assay after 6 Months of Storage at 0 °C

	1998 season			1999 season		
		mean scald score			mean scald score	
	dose, μmol/cup	DPA treated	control	dose, μmol/cup	DPA treated	control
control	0	0.07	3.27[a]	0	0.13	3.37[a]
	0	0.00	3.57[a]	0	0.23	3.33[a]
farnesyl hydroperoxide **2a**	2.6	0.07[b]	2.59[b]	3.52	0.27	3.23
	0.52	0.30[b]***	2.37[b]*	0.71	0.30	3.17
trienol **2b**	2.6	1.17[b]***	3.97[b]	2.98	0.83[b]***	3.30[b]
	0.52	0.30[b]***	2.76[b]	0.60	0.33[b]	2.33[b]*
cumyl hydroperoxide **4**	not tested			1.85	0.37	3.40
				0.37	0.47	3.20

[a] Significant difference from DPA control treatment $P < 0.001$. [b] Scores within treatments are significantly different $P < 0.05$. An asterisk indicates significant difference from appropriate DPA control $P < 0.05$; three asterisks, $P < 0.001$ by Fischer's exact test.

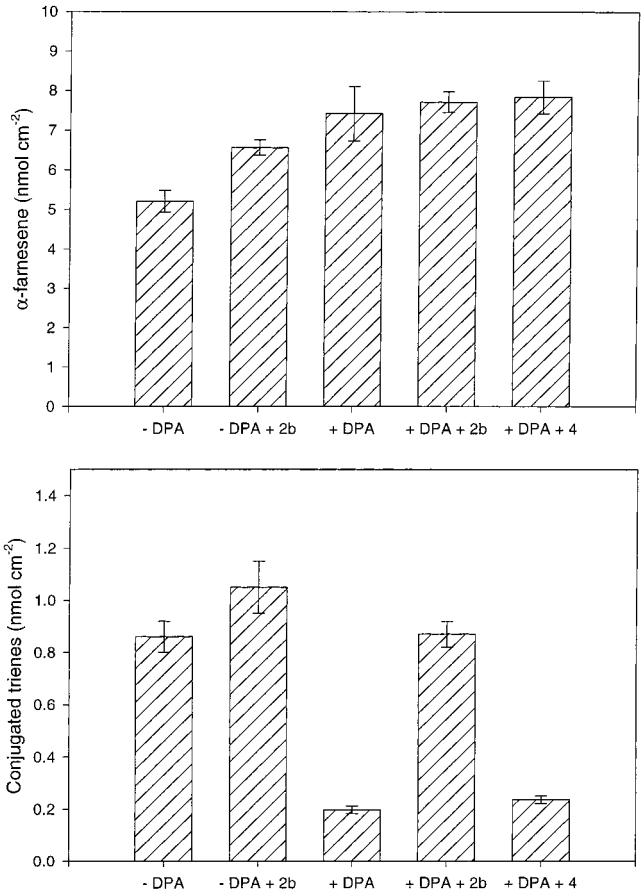

Figure 3. Effect of treatment with diphenylamine, trienol **2b** (2.98 μmol/cup for DPA treated and 0.6 μmol/cup for DPA control) and cumyl hydroperoxide **4** (1.85 μmol/cup) on concentrations (mean ± SEM) of α-farnesene and conjugated trienes in Granny Smith apples (1999 harvest season).

Microscopic examination of scalded tissue from the biossays and of naturally occurring scald showed indistinguishable symptoms of cell browning and collapse (*2*).

Effect of Diphenylamine Treatment on Activity of Conjugated Trienes. Diphenylamine is widely used as an antioxidant to control the development of superficial scald in stored apples (*5*, *40*, *54*). It was hypothesised that if application of conjugated trienes to fruit caused scald symptoms by the same mechanism as natural scald, then symptoms should also be inhibited by diphenylamine.

Table 3. Structure−Activity Relationships of Conjugated Trienes and Analogues (1998 Harvest Year) on Scald Intensity in Granny Smith Apples after 6 Months of Storage at 0 °C using the Vapor Diffusion Assay

treatment	μmol of compound added to cup	mean scald score
control	0.0	2.27
	0.0	2.33
farnesyl hydroperoxide **2a**	2.59	3.07
	0.52	3.73***
trienol **2b**	2.57	4.37[a]***
	0.52	3.73[a]***
dehydronerolidol **5**	2.64	4.80[a]***
	0.53	3.93[a]***
nerolidol **6**	2.59	3.23
	0.52	3.47**
tetraene **7**	2.62	2.60
	0.53	3.20**
cumyl hydroperoxide **4**	2.60	3.23[b]*
	0.52	2.97
hydrogen peroxide	45	3.33*
	4.5	3.47**
2-phenylpropan-2-ol	2.64	2.40
	0.53	2.87

[a] Scores within treatments are significantly different $P < 0.05$. An asterisk indicates significant difference from control $P < 0.05$; two asterisks, $P < 0.01$; three asterisks, $P < 0.001$ by Fischer's exact test. [b] Dose-dependent small dark sunken pock marks about lenticels.

Early-season fruits (April 1998−March 1999) were treated with diphenylamine before exposure to micromole doses of **2a**, **2b**, and **4** (1999 only) in the diffusion cup bioassay (Table 2). Fruits were also treated with **2a**, **2b**, and **4** but not diphenylamine as additional controls. In each year, diphenylamine treatment significantly reduced the severity of superficial scald, increased levels of α-farnesene, and decreased levels of conjugated trienes in the apple wax at the end of the cold storage (Figure 3). All fruits without diphenylamine treatment showed higher, but comparable, levels of scald, such that no relevant statistical differences could be shown between treatments. The absence of any increase in levels of scald due to treatment with **2a, 2b**, or **4** was believed due to the high background incidence

of superficial scald observed on fruit over these 2 years. Exposure of diphenylamine-treated fruit to micromolar concentrations of farnesyl hydroperoxide **2a** gave a significant increase in scald severity on only one occasion (Table 2). As previously observed (Tables 1 and 3), lower doses of **2a** may produce greater scald symptoms. Possibly, **2a** is more stable at lower concentrations.

Exposure of diphenylamine-treated fruit to micromole amounts of trienol **2b** (Table 2) gave elevated levels of superficial scald in a dose-dependent manner. The extent of scald induction was, however, consistently lower than previously observed with similar doses of **2b** on fruit that had not been subjected to a diphenylamine treatment (Tables 1 and 3), suggesting an inhibition of the scald-inducing activity of **2b** by diphenylamine. Treatment of DPA-treated fruit with the highest dose of trienol **2b** (3 μg/cup) had no effect on the levels of α-farnesene in the fruit skin but did increase the level of conjugated trienes (Figure 3), accounting for 0.6% of the amount of **2b** initially added. Exposure of diphenylamine-treated fruit to cumyl hydroperoxide **4** at comparable concentrations did not produce a significant increase in scald symptoms, confirming the greater activity of trienol **2b** relative to **4** previously observed (Tables 1 and 2). That trienol **2b** can partially overcome the inhibition of scald produced by a diphenylamine pretreatment suggests these two compounds may act via a common mechanism.

Structure—Activity Relationships. To establish if scald-inducing activity was primarily related to the presence of hydroperoxide or conjugated triene moieties, some preliminary structure activity studies were carried out (Table 3). Treatment of fruit with vapor of either **4** or hydrogen peroxide increased scald scores, although both compounds appeared to be less active than **2a**. 2-Phenyl-2-propanol, the alcohol analogue of **4**, was inactive, confirming that scald-inducing activity was derived from the hydroperoxide functionality.

The most active conjugated triene in the diffusion assay was not the hydroperoxide **2a** but trienol **2b**. Significantly, dehydronerolidol **5**, the synthetic precursor to **2b** (Figure 2), showed equivalent high scald-inducing activity with symptoms indistinguishable from those of **2b**. Microscopic examination of scalded apple tissue treated with dehydronerolidol showed cell damage indistinguishable from that of tissue treated with trienol **2b** or of natural scald. Dehydronerolidol rearranges to **2b** under acid catalysis (*16*), and this is probably the source of this activity. Neither nerolidol **6** (not a precursor to trienol **2b**) nor tetraene **7** (the highly autoxidizable elimination product of trienol **2b**) showed comparable activity in the bioassay. Although the instability of hydroperoxide **2a** makes detailed comparisons difficult, the high molar activity of **2b** and of its synthetic precursor **5** suggests there are further structural requirements for scald-inducing activity beyond the presence of a hydroperoxide moiety.

We have shown that the conjugated trienes **2a** and **2b** can produce symptoms indistinguishable from those of superficial scald when applied to apples at concentrations comparable to those found in apple skin. The related compounds **3a** and **5** also produced scald symptoms. 6-Methyl-5-hepten-2-one has also been postulated as a possible causal agent of superficial scald (*24*, *51*) in addition to the α-farnesene oxidation products originally proposed by Anet (*13*). In our hands, treatment of apples with 6-methyl-5-hepten-2-one did not increase the incidence of superficial scald (unpublished experiments). 6-Methyl-5-hepten-2-one is, however, produced during the autoxidation of α-farnesene (*10*), hydroperoxide **2a** (*16*), and trienol **2b** (*17*) and may be a more sensitive indicator than the conjugated trienols of oxidative processes occurring in the cuticular wax (*51*). The physiological processes leading to the development of superficial scald are obviously complex and involve ripening (*26*), responses to chilling and stress (*25*, *37*), the balance of oxidative and antioxidative processes (*20*, *23*), and physical issues of movement and loss of scald-inducing compounds in and from the fruit (*24*, *51*, *55*). Although we cannot exclude the possibility that the scald-inducing activity of the compounds tested here arises from their in situ oxidation to 6-methyl-5-hepten-2-one, our results are consistent with the original proposal of Anet (*13*), that conjugated triene oxidation products of α-farnesene are the immediate causal agents of superficial scald.

ACKNOWLEDGMENT

We thank D. Hopcroft and R. Bennet for assistance with photography and light microscopy.

LITERATURE CITED

(1) Meigh, D. F. Apple scald. In *Food Science and Technology. The Biochemistry of Fruits and Their Products*; Hulme, A. C., Ed.; Academic: London, U.K., 1970; pp 555−569.
(2) Bain, J. M.; Mercer, F. V. The submicroscopic cytology of superficial scald, a physiological disease of apples. *Aust. J. Biol. Sci.* **1963**, *16*, 442−449.
(3) Bain, J. M. A. A histological study of the development of superficial scald in Granny Smith apples. *J. Hortic. Sci.* **1956**, *31*, 234−238.
(4) Huelin, F. E.; Coggiola, I. M. Superficial scald, a functional disorder of stored apples. VII. Effect of applied α-farnesene, temperature and diphenylamine on scald and the concentration and oxidation of α-farnesene in the fruit. *J. Sci. Food Agric.* **1970**, *21*, 584−589.
(5) Huelin, F. E.; Coggiola, I. M. Superficial scald, a functional disorder of stored apples. V. Oxidation of α- farnesene and its inhibition by diphenylamine. *J. Sci. Food Agric.* **1970**, *21*, 44−48.
(6) Meir, S.; Bramlage, W. J. Antioxidant activity in 'Cortland' apple peel and susceptibility to superficial scald after storage. *J. Am. Soc. Hortic. Sci.* **1988**, *113*, 412−418.
(7) Chen, P. M.; Varga, D. M.; Mielke, E. A.; Facteau, T. J.; Drake, S. R. Control of superficial scald on 'd'Anjou' pears by ethoxyquin: Oxidation of α-farnesene and its inhibition. *J. Food Sci.* **1990**, *55*, 171−180.
(8) Gallerani, G.; Pratella, G. C. Upgrading peroxide detection to prevent superficial scald of apples by HPLC. *Adv. Hortic. Sci.* **1991**, *5*, 139−143.
(9) Meigh, D. F.; Filmer, A. A. E. Natural skin coating of the apple and its influence on scald in storage III. α-Farnesene. *J. Sci. Food Agric.* **1969**, *20*, 139−143.
(10) Anet, E. F. L. J. Superficial scald, a functional disorder of stored apples. VIII. Volatile products from the autoxidation of α-farnesene. *J. Sci. Food Agric.* **1972**, *23*, 605−608.
(11) Mir, N. A.; Beaudry, R. Effect of superficial scald suppression by diphenylamine application on volatile evolution by stored Cortland apple fruit. *J. Agric. Food Chem.* **1999**, *47*, 7−11.
(12) Lurie, S.; Klein, J.; Ben-Arie, R. Physiological changes in diphenylamine-treated 'Granny Smith' apples. *Israel J. Bot.* **1989**, *38*, 199−207.
(13) Anet, E. F. L. J. Autoxidation of α-farnesene. *Aust. J. Chem.* **1969**, *22*, 2403−2410.

(14) Rowan, D. D.; Allen, J. M.; Fielder, S.; Spicer, J. A.; Brimble, M. A. Identification of conjugated triene oxidation products of α-farnesene in apple skin. *J. Agric. Food Chem*. **1995**, *43*, 2040−2045.
(15) Whitaker, B. D.; Solomos, T.; Harrison, D. J. Quantification of α- farnesene and its conjugated trienol oxidation products from apple peel by C18-HPLC with UV detection. *J. Agric. Food Chem*. **1997**, *45*, 760−765.
(16) Fielder, S.; Rowan, D. D.; Sherburn, M. S. Synthesis of polyene sesquiterpene hydroperoxides by regio- and stereoselective transposition reactions. *Tetrahedron* **1998**, 12907−12922.
(17) Whitaker, B. D.; Saftner, R. A. Temperature-dependent autoxidation of conjugated trienols from apple peel yields 6-methyl-5-hepten-2-one, a volatile implicated in induction of scald. *J. Agric. Food Chem*. **2000**, *48*, 2040−2043.
(18) Barden, C. L.; Bramlage, W. J. Accumulation of antioxidants in apple peel as related to preharvest factors and superficial scald susceptibility of the fruit. *J. Am. Soc. Hortic. Sci.* **1994**, *119*, 264−269.
(19) Abdallah, A. Y.; Gil, M. I.; Biasi, W.; Mitcham, E. J. Inhibition of superficial scald in apples by wounding: changes in lipids and phenolics. *Postharvest Biol. Technol*. **1997**, *12*, 203−212.
(20) Whitaker, B. D. Phenolic fatty-acid esters from the peel of 'Gala' apples and their possible role in resistance to superficial scald. *Postharvest Biol. Technol.* **1998**, *13*, 1−10.
(21) Du, Z.; Bramlage, W. J. A modified hypothesis on the role of conjugated trienes in superficial scald development on stored apples. *J. Am. Soc. Hortic. Sci.* **1993**, *118*, 807−813.
(22) Rao, M. V.; Watkins, C. B.; Brown, S. K.; Weeden, N. F. Active oxygen species metabolism in 'White Angel' × 'Rome Beauty' apple selections resistant and susceptible to superficial scald. *J. Am. Soc. Hortic. Sci.* **1998**, *123*, 299−304.
(23) Whitaker, B. D.; Nock, J. F.; Watkins, C. B. Peel tissue α-farnesene and conjugated trienol concentrations during storage of 'White Angel' × 'Rome Beauty' hybrid apple selections susceptible and resistant to superficial scald. *Postharvest Biol. Technol.* **2000**, *20*, 231−241.
(24) Song, J.; Beaudry, R. M. Rethinking apple scald: new hypothesis on the causal reason for development of scald in apples. Abstract 233. *HortScience* **1996**, *31*, 605.
(25) Watkins, C. B.; Bramlage, W. J.; Cregoe, B. A. Superficial scald of 'Granny Smith' apples is expressed as a typical chilling injury. *J. Am. Soc. Hortic. Sci.* **1995**, *120*, 88−94.
(26) Fan, W.; Mattheis, J. P. Development of apple superficial scald, soft core, core flush, and greasiness is reduced by MCP. *J. Agric. Food Chem*. **1999**, *47*, 3063−3068.
(27) Gong, Y.; Tian, S. Inhibitory effect of diazacyclopentadiene on the development of superficial scald in Granny Smith apples. *Plant Growth Regul.* **1998**, *26*, 117−121.
(28) Rupasinghe, H. P. V.; Murr, D. P.; Paliyath, G.; Skog, L. Inhibitory effect of 1-MCP on ripening and superficial scald development in 'McIntosh' and 'Delicious' apples. *J. Hortic. Sci. Biotechnol.* **2000**, *75*, 271−276.
(29) Watkins, C. B.; Nock, J. F.; Whitaker, B. D. Responses of early, mid and late season apple cultivars to postharvest application of 1-methylcyclopropene (1-MCP) under air and controlled atmosphere storage conditions. *Postharvest Biol. Technol.* **2000**, *19*, 17−32.
(30) Soria, Y.; Rescasens, I.; Gatius, F.; Puy, J. Multivariate analysis of superficial scald susceptibility on Granny Smith apples dipped with different postharvest treatments. *J. Agric. Food Chem*. **1999**, *47*, 4854−4858.
(31) Anet, E. F. L. J.; Coggiola, I. M. Superficial scald, a functional disorder of stored apples. X. Control of α-farnesene autoxidation. *J. Sci. Food Agric.* **1974**, *25*, 293−298.
(32) Ingle, M.; D'Souza, M. C. Physiology and control of superficial scald of apples: a review. *HortScience* **1989**, *24*, 28−31.
(33) Lurie, S.; Klein, J. D.; Ben-Arie, R. Prestorage heat treatment delays development of superficial scald on 'Granny Smith' apples. *HortScience* **1991**, *26*, 166−167.
(34) Wang, Z.; Dilley, D. R. Initial low oxygen stress controls superficial scald of apples. *Postharvest Biol. Technol.* **2000**, *18*, 201−213.
(35) Scott, K. J.; Yuen, C. M. C.; Ghahramani, F. Ethanol vapour—a new anti-scald treatment for apples. *Postharvest Biol. Technol.* **1995**, *6*, 201−208.
(36) Ghahramani, F.; Scott, K. J. The action of ethanol in controlling superficial scald of apples. *Aust. J. Agric. Res.* **1998**, *49*, 199−205.
(37) Ghahramani, F.; Scott, K. J. Oxygen stress of Granny Smith apples in relation to superficial scald, ethanol, α-farnesene and conjugated trienes. *Aust. J. Agric. Res.* **1998**, *49*, 207−210.
(38) Ghahramani, F.; Scott, K. J.; Buckle, K. A.; Paton, J. E. A comparison of the effects of ethanol and higher alcohols for the control of superficial scald in apples. *J. Hort. Sci. Biotechnol.* **1999**, *74*, 87−93.
(39) Bauchot, A. D.; John, P.; Soria, Y.; Recasens, I. Carbon dioxide, oxygen, and ethylene changes in relation to the development of scald in Granny Smith apples after cold storage. *J. Agric. Food Chem*. **1995**, *43*, 3007−3011.
(40) Whitaker, B. D. DPA alters α-farnesene metabolism in peel of 'Empire' apples stored in air or 1.5% O_2 atmosphere. *Postharvest Biol. Technol.* **2000**, *18*, 91−97.
(41) Fielder, S.; Rowan, D. D.; Sherburn, M. S. Synthesis of α-farnesene hydroperoxides. *Synlett* **1996**, 349−350.
(42) Huelin, F. E. Superficial scald, A functional disorder of stored apples. II. Promoters and inhibitors. *J. Sci. Food Agric.* **1964**, *15*, 227−236.
(43) Wills, R. B. H.; Scott, K. J.; McBailey, W. Reduction in superficial scald in apples with monoterpenes. *Aust. J. Agric. Res.* **1977**, *28*, 445−448.
(44) Huelin, F. E.; Coggiola, I. M. Superficial scald, a functional disorder of stored apples IV. Effect of variety, maturity, oiled wraps and diphenylamine on the concentration of α-farnesene in the fruit. *J. Sci. Food Agric.* **1968**, *19*, 297−301.
(45) Anet, E. F. L. J. Superficial scald, a functional disorder of stored apples. IX. Effect of maturity and ventilation. *J. Sci. Food Agric.* **1972**, *23*, 763−769.
(46) Wang, Z.; Dilley, D. R. Hypobaric storage removes scald-related volatiles during the low-temperature induction of superficial scald of apples. *Postharvest Biol. Technol.* **2000**, *18*, 191−199.
(47) Matich, A. J.; Banks, N. H.; Rowan, D. D. Modification of α-farnesene levels in cool-stored 'Granny Smith' apples by ventilation. *Postharvest Biol. Technol.* **1998**, *14*, 159−170.
(48) Fielder, S.; Rowan, D. D. The synthesis of d_6-α-farnesene. *J. Labelled Compd. Radiopharm*. **1994**, *34*, 1075−1085.
(49) Song, J.; Leepipattanawit, R.; Deng, W.; Beaudry, R. M. Hexanal vapor is a natural, metabolizable fungicide: Inhibition of fungal activity and enhancement of aroma biosynthesis in apple slices. *J. Am. Soc. Hortic. Sci.* **1996**, *121*, 937−942.
(50) Leepipattanawit, R.; Beaudry, R. M.; Hernandez, R. J. Control of decay in modified-atmosphere packages of sliced apples using 2-nonanone vapor. *J. Food Sci.* **1997**, *62*, 1043−1047.
(51) Mir, N.; Perez, R.; Beaudry, R. M. A poststorage burst of 6-methyl-5-hepten-2-one (MHO) may be related to superficial scald in 'Cortland' apples. *J. Am. Soc. Hortic. Sci.* **1999**, *124*, 173−176.
(52) Merck. *The Merck Index*, 11th ed.; Merck and Co. Inc.: Rahay, NJ, 1989; p 1383.

(53) Rowan, D. D.; Allen, J. M.; Fielder, S.; Hunt, M. B. Biosynthesis of straight-chain ester volatiles in Red Delicious and Granny Smith apples using deuterium-labeled precursors. *J. Agric. Food Chem*. **1999**, *47*, 2553−2562.

(54) Ingle, M.; D'Souza, M. C.; Morris, J. C. *A Comparison of Inhibitor Treatment Systems for Controlling Superficial Scald on Four Apple Cultivars*; West Virginia University: Morgantown, WV, 1990; Vol. 704.

(55) Wee, S.; Beaudry, R. M. Volatile cuticular components involved in superficial scald. *HortScience* **1996**, *31*, 605.

Received for review December 27, 2000. Revised manuscript received March 28, 2001. Accepted March 29, 2001. We thank the New Zealand Foundation for Research, Science and Technology for financial support.

JF0015221