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Antifungal compounds from idioblast cells isolated from avocado fruits

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Abstract

(*E,Z,Z*)-1-Acetoxy-2-hydroxy-4-oxo-heneicosa-5,12,15-triene was isolated from avocado, *Persea americana* Mill., idioblast cells. It inhibited spore germination of the fungal pathogen *Colletotrichum gloeosporioides*. Full characterization is also reported for two additional compounds that have been described and partially characterized previously.

Keywords: *Persea americana*; Lauraceae; Avocado; Idioblast cell; Diene; Persin; Antifungal compounds

1. Introduction

Avocado fruits, *Persea americana* Mill., contain specialized idioblast cells that are almost completely filled with an oil containing alkaloids, sesquiterpene hydroperoxides and possibly other terpenes (Platt and Thomson, 1992). Several of these constituents inhibit the in vitro vegetative growth of the avocado fungal pathogen *Colletotrichum gloeosporioides* (Sivanathan and Adikaram, 1989). The most active constituent termed "persin" (Oelrichs et al., 1995), was characterized as (*Z,Z*)-1-acetoxy-2-hydroxy-4-oxo-heneicosa-12,15-diene (Prusky et al., 1982). This antifungal diene possesses many other biological activities, as it is a growth inhibitor of the silkworm larvae *Bombyx mori* L. (Chang et al., 1975), an inducer of lactating mammary necrosis of mice (Rodriguez-Saona et al., 1997) as well as a feeding deterrent to larvae of *S. exiga* (Kashman et al., 1969).

Here we report on the isolation and characterization from idioblast cell oil of (*E,Z,Z*)-1-acetoxy-2-hydroxy-4-oxo-heneicosa-5,12,15-triene. This compound has antifungal activity, as it inhibits *C. gloeosporioides* germination and germ tube elongation similarly to the diene, persin. We also report the complete characterization of 1-acetoxy-2,4-dihydroxy-I-*N*-heptadeca-16-yne which had previously been incompletely described (Kashman et al., 1969).

2. Results and discussion

The HPLC profile of the idioblast crude extract is shown in Fig. 1A. Four major compounds (**1, 2, 3** and **4**), respectively, eluting at 6.05, 12.06, 19.75 and 25.96 min were consistently present, whereas other peaks varied in intensity according to the preparation examined. APCI (+) mass spectral analysis (Fig. 1B) showed that compounds **1** and **2** displayed the same fragmentation pattern, except that **2** was two mass units larger than **1**. A comparable observation was made with compounds **3** and **4**. The two pairs of compounds (**1, 2** and **3, 4**)

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were separated when the crude extracts were partitioned using silica gel flash-chromatography (data not shown); compounds **3** and **4** were eluted using CH_2Cl_2–EtOAc (85:15) as solvents whereas compounds **1** and **2** required CH_2Cl_2–EtOAc (60:40) for elution.

The four compounds were purified to homogeneity using HPLC equipped with a refractive index detector and identified as described below.

An acetoxy group in compound **1** was evidenced by the presence of a singlet (3H) at 2.08 ppm in the ^{1}H-NMR spectrum, a quaternary carbon at 171.35 ppm in the ^{13}C-NMR spectrum and an absorbance maximum at 1730 cm^{-1} in the infrared spectrum. An IR maxi-

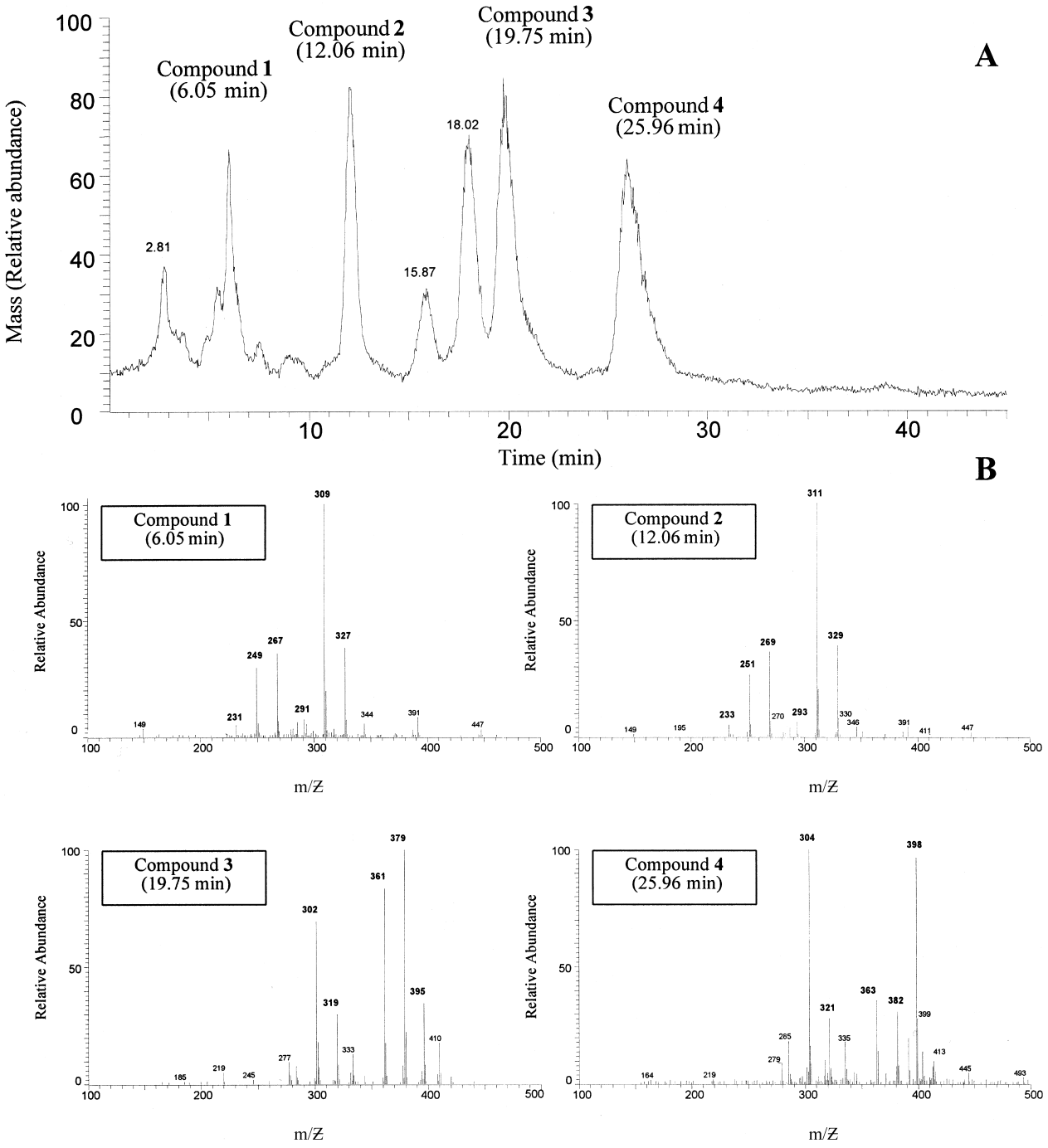


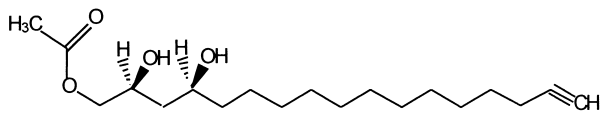
Fig. 1. HPLC separation (A) and MS fragmentation (B) of compounds **1–4**. Crude extracts were separated on HPLC using a reversed-phase column (25 × 0.5 cm Beckman ODS ultrasphere 5 μm column) and methanol–water (4:1) as solvent with a flow rate of 1.5 ml/min. The HPLC eluant was monitored with APCI(+) mass spectrometric detection.

mum at 3430 cm^{-1} together with two broad singlets at 2.63 and 3.44 (D_2O exchangeable) in the ^{1}H-NMR spectrum indicated the presence of two hydroxy groups. The existence of these three functional groups was supported by the mass spectrum data (Fig. 1B) showing the presence of the following molecular ions (at m/z): $[M+H]^+$ (327), $[M+H-H_2O]^+$ (309), $[M+H-2H_2O]^+$ (291), $[M+H-HOAc]^+$ (267), $[M+H-HOAc-H_2O]^+$ (249) and $[M+H-HOAc-2H_2O]^+$ (231), respectively. ^{1}H-NMR spectroscopic data indicated the presence of a long hydrocarbon chain (a multiplet at 1.24 ppm representing 7 CH_2), while carbon NMR spectral data showed that the molecule contained 19 carbon atoms. Consequently, the molecular formula of compound **1** was assigned as $C_{19}H_{34}O_4$.

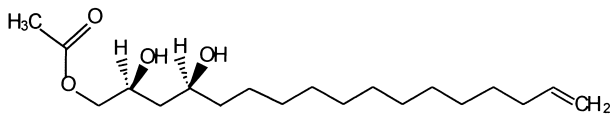
Comparable observations to those above were made for compound **2**, which only differed by the presence of two additional mass units in its aliphatic chain, according to its fragmentation pattern (Fig. 1B). Consequently, the formula of compound **2** was $C_{19}H_{36}O_4$.

Further analysis revealed that the only difference between compounds **1** and **2** was in the degree of unsaturation. IR and ^{1}H-NMR spectra revealed that compound **1** contained a terminal acetylenic group (IR maximum at 3290 cm^{-1} and an alkynyl proton at 1.91 ppm), whereas compound **2** had a vinylic group (IR maxima at 995 and 910 cm^{-1} and three typical vinylic protons at 5.82, 4.99 and 4.93 ppm). These terminal unsaturations were supported by the absence of any terminal methyl group in the ^{1}H and ^{13}C spectra of both compounds **1** and **2**. From the above data, the structures 1-acetoxy-2,4-dihydroxy-*N*-heptadeca-16-yne and 1-acetoxy-2,4-dihydroxy-*N*-heptadeca-16-ene were assigned for compounds **1** and **2**, respectively (Fig. 2). All the NMR spectral assignments for these structures were confirmed by analysis of their DEPT and 2D-NMR (COSY, HMBC, HMQC) spectra. The complete ^{1}H- and ^{13}C-NMR assignments of both compounds **1** and **2** are reported in Table 1. For both compounds, the ^{13}C and ^{1}H assignments in the region C1–C5 were broadened due to the exchange of the acetoxy group between the hydroxyls on carbons 1, 2 and 4.

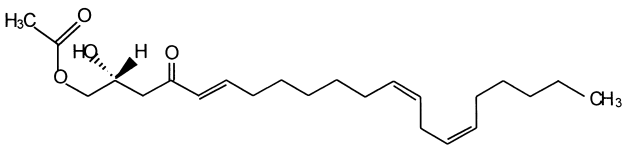
Note that **1** and **2** from avocado fruit were previously partially characterized (Brown, 1972; Kashman et al., 1970). Our experiments provide the first ^{13}C data and complete molecular characterization of compound **1** (Table 1). The absolute configuration of each asymmetric center of compound **1** (C2 and C4) was reported to be S (Kashman et al., 1970). The optical rotation of the purified compound was $[\alpha]_D^{22}$ -2.7° ($CHCL_3$, c 0.24). This low rotation was in good agreement with C2 and C4 having the same absolute configuration. The involvement of compound **2** in the



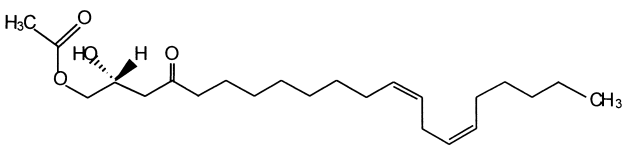
1-acetoxy-2,4-dihydroxy-*n*-heptadeca-16-yne, **1**



1-acetoxy-2,4-dihydroxy-*n*-heptadeca-16-ene, **2**



(*Z, Z, E*)-1-acetoxy-2-hydroxy-4-oxo-heneicosa-5,12,15-triene, **3**



(*Z, Z*) -1-acetoxy-2-hydroxy-4-oxo-heneicosa-12,15-diene, **4**

Fig. 2. Compounds **1–4**.

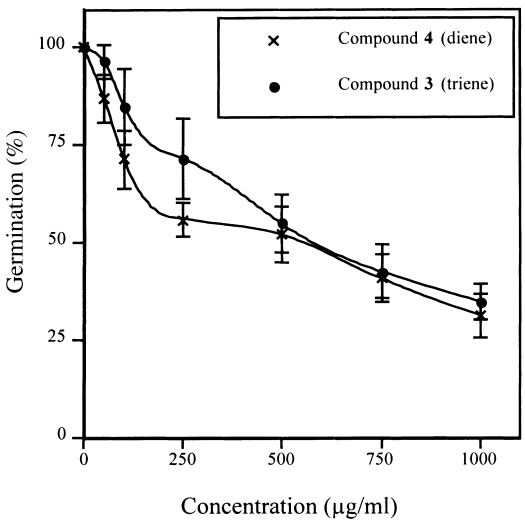


Fig. 3. Inhibition of *C. gloeosporioides* germination. Biological activity of both compounds towards *C. gloeosporioides* was measured on 13 mm-diameter filters (0.45 µm, Millipore) according to Prusky et al. (1982). The activity of each compound was determined by measuring the percent of spore germination and germ tube elongation (considered inhibited when length was less than twice the length of the spore).

quiescence of the germinated appressoria of *C. gloeosporioides* in unripe avocado was recently described (Prusky et al., 1991a). It has also been reported as an inhibitor of soybean callus growth and wheat coleoptiles elongation (Bittner et al., 1971). The optical rotation measured for compound **2** was $[\alpha]_D^{22}$ -2.5° ($CHCl_3$, *c* 0.89) in good agreement with that reported by Kashman et al. (1969).

Our IR and NMR spectroscopic analysis of compound **4** yielded data (not shown) in close agreement with those of Oelrichs et al. (1995) for persin, (*Z,Z*)-1-acetoxy-2-hydroxy-4-oxo-heneicosa-12,15-diene (Fig. 3). This compound has been fully characterized in recent publications studying the toxicity of idioblast cell oil (Oelrichs et al., 1995; Prusky et al., 1982; Rodriguez-Saona et al., 1997). Its chemical synthesis determined the absolute configuration of its chiral center (C2) to be *R* (Bull and Carman, 1994). The optical rotation ($[\alpha]_D^{22}$ $+11.1^\circ$; $CHCl_3$, *c* 0.74) was also in good agreement with values previously published (Chang et al., 1975; Oelirichs et al., 1995).

The same functionalities as those reported for compound **4** (two *cis* non-conjugated double bonds, a keto, a hydroxy and an acetate group), were also present in compound **3**. The presence of these groups was in agreement with the fragmentation pattern reported in Fig. 1B, which showed the presence of the following molecular ions (at *m/z*): $[M+H]^+$ (379), $[M+H-H_2O]^+$ (361), $[M+H-HOAc]^+$ (319) and $[M+H-HOAc-H_2O]^+$ (301). Similar to compounds **1** and **2**, compounds **3** and **4** only differed by the presence in compound **3** of an additional unsaturation in the aliphatic part of the molecule (Fig. 1B). The molecular formula was consequently assigned as $C_{23}H_{38}O_4$. This result was supported by the observation in Fig. 1B of a molecular ion (rel. int.) at *m/z* 396 $[M+NH_4]^+$ (33). The presence in the UV spectrum of compound **3** of an absorption peak at 227 nm suggested that the additional double bond was conjugated (data not shown). The ^{13}C-NMR spectrum showed that the carbon resonance of the keto group (C4) was shifted to higher field than that in compound **4**, indicating that the additional double bond was conjugated to the keto group, i.e. between C5 and C6. The configuration of this double bond was established to be *trans* based on the 16 Hz coupling constant between H5 and H6 in the ^{1}H-NMR spectrum, and the absorptions at 960 and 980 cm^{-1} in the IR spectrum. Consequently, the structure of compound **3** was (*E,Z,Z*)-1-acetoxy-2-hydroxy-4-oxo-heneicosa-5,12,15-

Table 1
^{1}H- and ^{13}C-NMR spectral data for compounds **1** and **2**[a]

Carbon atom	Compound **1** (acetylene)			Compound **2** (vinyl)		
	^{13}C Chemical shift (ppm)	^{1}H Chemical shift (ppm)	Coupling constants (Hz)	^{13}C Chemical shift (ppm)	^{1}H Chemical shift (ppm)	Coupling constants (Hz)
C1	68.82	4.09 *m* 3.97 *m*		68.51	4.11 *m* 4.00 *m*	
C2	71.08	4.09 *m*		70.75	4.11 *m*	
C3	39.33	1.56 *m*		38.98	1.60 *m*	
C4	72.79	3.87 *m*		72.49	3.89 *m*	
C5	38.49	1.44 *m*		38.18	1.47 *m*	
C6	25.63	1.24 *m*		25.34	1.27 *m*	
C7	29.90[b]	1.24 *m*		28.98[c]	1.27 *m*	
C8	29.89[b]	1.24 *m*		29.18[c]	1.27 *m*	
C9	29.89[b]	1.24 *m*		29.54[c]	1.27 *m*	
C10	29.87[b]	1.24 *m*		29.62[c]	1.27 *m*	
C11	29.81[b]	1.24 *m*		29.62[c]	1.27 *m*	
C12	29.43[b]	1.24 *m*		29.62[c]	1.27 *m*	
C13	29.08	1.48 *m*		29.54[c]	1.27 *m*	
C14	28.82	1.36 *m*		29.18	1.37 *m*	
C15	18.75	2.15 *dt*	J = 7.3, 2.7	33.85	2.04 *br q*	J = 7
C16	68.31	–		139.14	5.82 *ddt*	J = 17, 10.5, 6.7
C17	85.06	1.91 *t*	J = 2.7	114.01	4.93 *ddt* 4.99 *ddt*	J = 10.5, 2.1, 1.2 J = 17, 1.6, 1.2
Cα	171.35	–		171.09	–	
Cβ	21.45	2.08 *s*		20.96	2.09 *s*	
OH	–	2.63 *br s*		–	2.96 *br s*	
OH	–	3.44 *br s*		–	3.58 *br s*	

[a] Solvent $CDCl_3$; Chemical shift from TMS.
[b,c] Values are interchangeable.

triene (Fig. 3). The complete ^{1}H- and ^{13}C-NMR assignment of compound **3** is reported in Table 2. ^{1}H–^{1}H coupling constants were deduced by simulation of the various strongly coupled spin systems in an iterative fashion, and then subtracting the results from the experimental spectrum. Degeneracy of the ^{1}H chemical shift for the *cis* double bonds made the determination of these coupling constants impossible. The optical rotation we measured for compound **3** was $[\alpha]_D^{22}$ $+11.7^{\circ}$ ($CHCI_3$, *c* 0.22) indicating that its chiral center (C2) had the same absolute configuration as that of the diene (R-configuration). Although this compound is very similar to the diene, it has thus far never been described. Compound **3** was also shown to be present in fruit pericarp and in leaves of both Hass and Fuerte avocado cultivars.

As compounds **3** and **4** were very similar, and as the latter diene inhibits spore germination and germ tube elongation of *C. gloeosporioides* (Prusky et al., 1991b), both compounds were next compared for antifungal activity. Fig. 3 shows that both compounds **3** and **4** had inhibitory effects on fungal growth. At concentrations lower than 500 μg/ml, the diene was a stronger inhibitor than compound **3**. At higher concentrations, both compounds were similarly active, and an ED_{50} of about 600 μg/ml was obtained for each. The ED_{50} of the diene reported in this study is higher than that reported previously (ED_{50} = 450 μg/ml, Prusky et al. (1982)). Nevertheless, this value, together with the variations of the level of the antifungal diene present during fruit ripening, support its involvement in the quiescent infection of unripe avocados by *C. gloeosporioides* (Prusky et al., 1991b).

It was previously hypothesized that the biological activity of the diene could be related to its chemical similarity to the monoglyceride of linoleate (Bull and Carman, 1994). The new compound reported in this study also presents such a similarity, and the presence of a *trans* double bond in such a lipid like molecule is particularly interesting. Its possible involvement in the quiescence of *C. gloeosporioides* in unripe avocado fruit is presently under investigation.

Table 2
^{1}H- and ^{13}C-NMR spectral data for compound 3[a]

Carbon atom	^{13}C Chemical shift (ppm)	^{1}H Chemical shift (ppm)	Coupling constants (Hz)	HMBC cross peaks[b]
C1	67.30	4.12 *m*	$J_{1a,2}$ = 4.0; $J_{1a,1b}$ = −11.3	Cα(s), C2(w), C3(s)
		4.07 *m*	$J_{1b,2}$ = 6.1; $J_{1b,1a}$ = −11.3	Cα(s), C2(s), C3(s)
C2	66.15	4.32 *m*	$J_{2,1a}$ = $J_{2,3b}$ = 4.0; $J_{2,1b}$ = 6.1; $J_{2,3a}$ = 8.0; $J_{2,OH}$ = 0.5	C1(w)
C3	42.30	2.75 *m*	$J_{3a,2}$ = 8.0; $J_{3a,3b}$ = −18.0	C1(s), C2(s), C4(s), C5(s)
		2.73 *m*	$J_{3b,2}$ = 4.0; $J_{3b,3a}$ = −18.0	C1(s), C2(s), C4(s), C5(s)
C4	199.45	–		
C5	130.19[e]	6.09 *dt*	$J_{5,6}$ = 16.0; $J_{5,7a}$ = $J_{5,7b}$ = 1.5	C3(w), C4(s), C6(w), C7(s)
C6	149.26	6.87 *dt*	$J_{6,5}$ = 16.0; $J_{6,7a}$ = $J_{6,7b}$ = 7.0	C4 (s),C5(w), C7(s), C8(s)
C7	32.61	2.22 *q*	$J_{7a,6}$ = $J_{7b,6}$ = 7.0; $J_{7a,5}$ = $J_{7b,5}$ = 1.5	C5(s), C6(s), C8(s)
C8	27.97	1.46 *p*	J = 7.3	C6(w), C7(w)
C9	29.44[c]	1.30 *m*		
C10	29.40[c]	1.30 *m*		
C11	27.28[d]	2.03 *q*	J = 6.5	
C12	130.17[e]	5.33 *m*		
C13	129.58[e]	5.33 *m*		
C14	25.71	2.75 *t*	J = 6.0	C12(s), C13(s), C15(s), C16(s)
C15	128.21[e]	5.33 *m*		
C16	127.66[e]	5.33 *m*		
C17	27.11[d]	2.03 *q*	J = 6.5	
C18	28.90[a]	1.30 *m*		
C19	31.59	1.30 *m*		
C20	22.66	1.30 *m*		
C21	14.19	0.87 *t*		C19(s), C20(s)
Cα	170.83	–		
Cβ	20.99	2.09 *s*		Cα(s)
OH	–	3.30 *br s*	$J_{OH,2}$ = 0.5	C1(w), C2(w)

[a] Solvent $CDCl_3$; Chemical shift from TMS.
[b] s = strong, w = weak.
[c,d,e] Values are interchangeable.

3. Experimental

3.1. General

MS data were obtained using a LCQ Finnigan Thermoquest mass spectrometer with ion trap detector, positive atmospheric pressure chemical ionization (APCI+) and the following parameters: discharge current 5 μA, capillary voltage 8 V, capillary temperature 150°C, vaporizing temperature 450°C and sheath gas flow 6 l/min. All other parameters were optimized for compound **4** (diene) by the software. The mass spectrometer was coupled to a Waters Alliance 2690 HPLC system (1.5 ml/min, 80% methanol/20% water). NMR spectra were recorded in $CDCl_3$ solution with TMS as internal standard on a Varian Mercury 300 MHz Spectrometer (300.1 MHz for ^1H and 75.5 MHz for ^{13}C) and on a Varian Inova 500 MHz Spectrometer (499.8 MHz for ^1H and 125.7 MHz for ^{13}C) at 22°C. All 2D NMR data were recorded using standard pulse programs. IR spectra were measured using a Paragon 1000 FT-IR Spectrometer from Perkin-Elmer. Optical rotations were measured using a DIP-181 Digital Polarimeter (JASCO).

3.2. Plant material

Immature avocado fruits (120–180 days after fruit set) were obtained from trees of the cultivar Fuerte grown in the orchards at the University of California, Riverside.

3.3. Isolation of idioblast cells

Idioblast cells were isolated from avocado mesocarp according to the method of Platt and Thomson (1992) with slight modifications. Fifty grams of avocado mesocarp, 1–2 mm sliced, were incubated overnight in 100 ml of 10 mM MES buffer pH 5.5 containing 100 mM sorbitol, 1 mM $CaCl_2$, 0.2% DTT, 0.2% BSA, 1.5% Cellulozyme and 0.25% Macerozyme (both from YAKULT Honsha, Tokyo, Japan). The macerate was very briefly homogenized with a Waring blender, filtered through a 300 μm nylon mesh, and the resulting filtrate was further filtered through a 48 μm nylon mesh. The unfiltered residue, which contained the isolated idioblast cells, was washed twice with distilled water, and the cells were pelleted by centrifugation (10 min, 12,000 × *g*).

3.4. Isolation of compounds *1–4*

Isolated idioblast cells were homogenized in EtOH–H_2O (95:5) with a Polytron homogenizer and the homogenate was filtered through Miracloth. The filtrate was concentrated five-fold under vacuum at 40°C and extracted twice with an equivalent volume of dichloromethane. The organic layers were pooled and dried under vacuum. The residue was dissolved in 5 ml hexane and used as the crude extract.

When compounds were extracted from leaves, mesocarp and pericarp from fruits, plant material was first cut into thin slices with a razor blade and homogenized in ethanol 95% (2.5 ml/g) with a Waring blender before undergoing the above extraction procedure.

3.5. Purification of the compounds

The crude extract was either fractionated by flash chromatography or directly used for HPLC. For flash chromatography, a 25 ml column of silica gel type H (10–40 μM) was pre-equilibrated with 50 ml hexane, washed with 100 ml dichloromethane and eluted with 100ml dichloromethane containing increasing concentrations of ethyl acetate. For HPLC, a reversed-phase column (25 × 0.5 cm Beckman ODS ultrasphere 5 μm column) was used with EtOH–H_2O (4:1) as eluant at a flow rate of 1.5 ml/min. HPLC was monitored with UV detection ($\lambda = 205$ nm) and with either a refractive index detector for peak collection or an APCI (+) mass spectrometer detection for analysis.

3.5.1. Compound 1

$[\alpha]_D^{22}$ −2.7° ($CHCL_3$, *c* 0.24); IR ($CHCL_3$) ν_{max} cm^{-1}: 3430 *s* (OH); 3290 *s*, 2115 *w* (C ≡ CH); 1730 *s*, 1265 *s* (–CO–O–); 1460 *m*, 1370 *m* (CH_3–CO–O–); CIMS (APCI+) *m*/*z* (rel. int.): 344 (6), 327 $[M+H]^+$ (40), 309 $[M+H-H_2O]^+$ (100), 291 $[M+H-2H_2O]^+$ (7), 287 (7), 267 $[M+H-HOAc]^+$ (41), 249 $[M+H-HOAc-H_2O]^+$ (35), 231 $[M+H-HOAc-2H_2O]^+$ (6), 149 (4), ^1H- and ^{13}C-NMR spectral data are reported in Table 1.

3.5.2. Compound 2

$[\alpha]_D^{22}$ −2.5° ($CHCl_3$, *c* 0.89); IR ($CHCl_3$) ν_{max} cm^{-1}: 3420 *s* (OH); 3060 *w*, 1640 *w*, 995 *m*, 910 *m* (C||CH); 1740 *s*, 1265 *s* (–CO–O–); 1460 *m*, 1370 *m* (CH_3–CO–O–); CIMS (APCI+) *m*/*z* (rel. int.): 351 (2), 346 (4), 329 $[M+H]^+$ (39), 311 $[M+H-H_2O]^+$ (100), 293 $[M+H-2H_2O]^+$ (7), 287 (4), 269 $[M+H-HOAc]^+$ (37), 251 $[M+H-HOAc-H_2O]^+$ (27), 233 $[M+H-HOAc-2H_2O]^+$ (5); ^1H- and ^{13}C-NMR spectral data are reported in Table 1.

3.5.3. Compound 3

$[\alpha]_D^{22}$ +11.7° ($CHCl_3$, *c* 0.22); IR ($CHCl_3$) ν_{max} cm^{-1}: 3490 *s* (OH); 1660 *m*, 1630 *m* (C||C, *Z*); 980 *m*, 960 *m* (C||C, *E*); 1730 *s*, 1265 *s* (–CO–O–); 1460 *m*, 1380 *m* (CH_3–CO–O–); CIMS (APCI+) *m*/*z* (rel. int.): 410 (18), 396 $[M+NH_4]^+$ (33), 379 $[M+H]^+$ (100), 361 $[M+H-H_2O]^+$ (84), 333 (14), 319 $[M+H-HOAc]^+$ (30), 301 $[M+H-HOAc-H_2O]^+$

(70), 284 (8), 277 (10), 219 (5); ^{1}H- and ^{13}C-NMR spectral data are reported in Table 2.

3.6. Bioassay for antifungal activity

For both (*Z,Z*)-1-acetoxy-2-hydroxy-4-oxo-heneicosa-12,15-diene and (*E,Z,Z*)-1-acetoxy-2-hydroxy-4-oxo-heneicosa-5,12,15-triene, biological activity towards *C. gloeosporioides* was measured on 13 mm-diameter filters (0.45 μm, Millipore) according to Prusky et al. (1982). The activity of each compound was determined by measuring the percent of spore germination and germ tube elongation (considered inhibited when length was less than twice the length of the spore).

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