The inhibition of the fungus *Botrytis cinerea* by an eremophilane phytoalexin analogue

Isidro G. Collado[a]*, Athina Deligeorgopoulou[b], James R. Hanson[b]*, Peter B. Hitchcock[b] and Antonio J.Macias-Sanchez[a]

[a]*Departamento de Quimica Organica, Facultad de Ciencias, Universidad de Cadiz, Apartado 40, 11510 Puerto Real, Cadiz, Spain*

[b]*Department of Chemistry, University of Sussex, Brighton, Sussex BN1 9QJ, UK*

1α,11-Dihydroxy-4α,5α,7β,10β (H)-eremophilane **6** has been examined as a phytoalexin analogue and shown to be a powerful inhibitor of the growth of the plant pathogen, *Botrytis cinerea*. Its metabolites have been identified.

Keywords: eremophilanes, *Botrytis cinerea*, fungistatic action

The plant pathogenic fungus, *Botrytis cinerea* can cause serious economic losses of a number of crops such as tomatoes, lettuces and grapes.[1] There is a need to develop new strategies for the control of *B. cinerea* because some strains of the fungus have developed resistance to commercial fungicides[2] and because of the persistance of some fungicides in the food chain.[3,4] One strategy which we have examined[5] has been to use botryane biosynthetic mimics as fungistatic agents. Another strategy is to develop phytoalexin analogues.[6]

A number of eremophilane sesquiterpenoids, exemplified by capsidiol **1**, are produced by plants as phytoalexins in response to fungal attack. Some eremophilanes are found in foodstuffs and in order to minimise potential toxicity problems, these formed the starting materials for this investigation. Valencene **2**[7] and nootkatone**3**[8] are obtained from the skin of citrus fruits and we have converted them into capsidiol analogues. The substrates **5**,**6** and **8a** were prepared following previously described procedures.[9–12]

Oxymercuration of valencene **2** afforded a mixture of 10β,11-epoxy-4α,5α,7β-eremophilane and 11-hydroxy-4α,5α,7β-eremophil-1(10)-ene, valerianol, **4**.[9,10] The yield of the latter was increased to 60% by carrying out the reaction at 35°C for 72 h. Hydroboration of valerianol **4** followed by oxidation of the borane with alkaline hydrogen peroxide and then oxidation of the epimeric mixture of alcohols with chromium trioxide, gave the 1-ketone **5**. Reduction of this with sodium borohydride in methanol gave 1α,11-dihydroxy-4α,5α,7β,10β(H)-eremophilane **6**. The structure and stereochemistry of this alcohol and hence that of **5**, was confirmed by X-ray crystallography (see Fig. 1).

Reduction of nootkatone **3** with sodium borohydride gave 2α-hydroxy-4α,5α,7β-eremophil-1(10),11-diene **7**[11] which on oxymercuration, gave the cyclic ether, 2α-hydroxy-10β, 11-epoxy-4α,5α,7β-eremophilane **8a**.

The compounds **5**, **6** and **8a** were examined as inhibitors of the growth of the fungus *B.cinerea* (see Table 1). 1α, 11-Dihydroxy-4α,5α,7β,10β(H)-eremophilane **6** was a powerful inhibitor of the growth of *B.cinerea* whilst the 1-ketone **5** inhibited the growth to a lesser extent. The 2α-hydroxy-10β, 11-epoxy-4α,5α,7β-eremophilane **8a** was a poor inhibitor.

Since it was a powerful inhibitor of the growth of the fungus, incubation of 1α,11-dihydroxy-4α,5α,7β,10β(H)-eremophilane **6** with *B.cinerea* for 7 days only gave a low yield of two metabolites. The first was the 1-ketone **5** which was identified by comparison with the synthetic sample. The second was a secondary alcohol **9**. Its structure was established by X-ray crystallography (see Fig. 2) as 1β, 11-dihydroxy-4α,5α,7β,10α(H)-eremophilane. This showed that the stereochemistry had been inverted at both C-1 and C-10. Comparison of the structures of the starting material **6** and this metabolite shows that formation of the *cis* ring

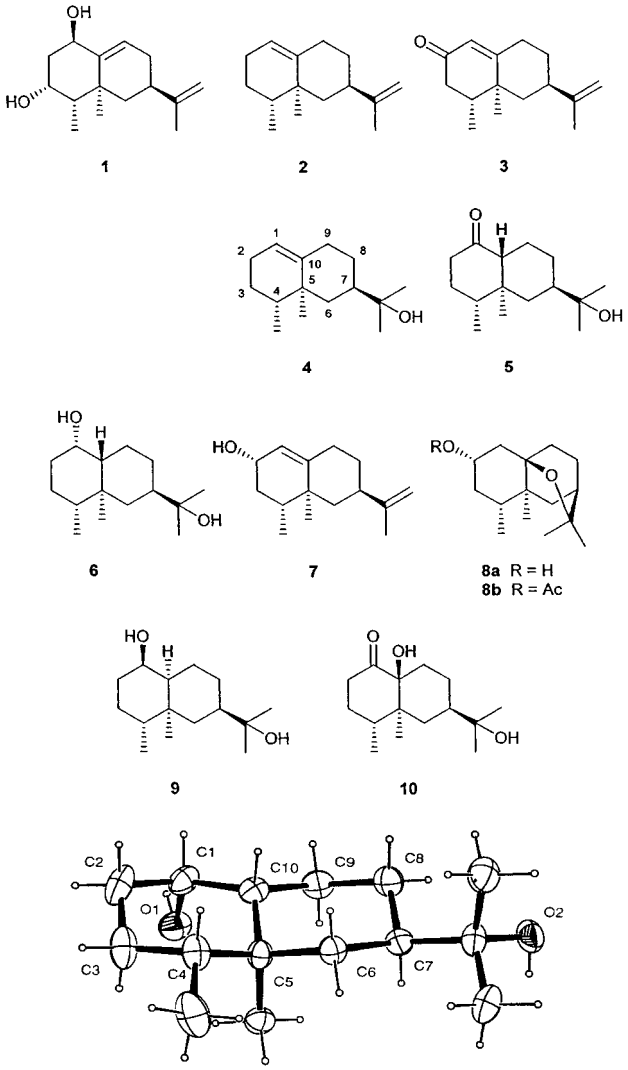

Fig 1 X-ray crystal structure of compound **6**.

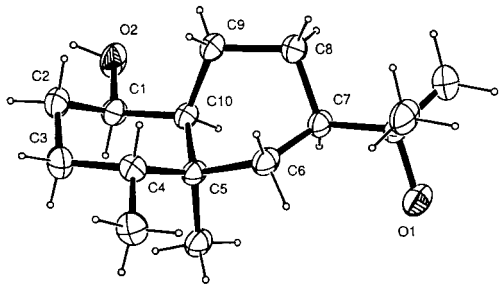

Fig 2 X-ray crystal structure of compound **9**.

* Correspondence

Table 1 Inhibition of *B. cinerea* by eremophilanes

% decrease in diameter of *B.cinerea* substrate / diameter of *B.cinerea* control vs time	Time/h				
	24	48	72	96	120
50ppm					
5	0	0	0	–	–
6	100	75	86	89	92
100ppm					
5	100	100	50	75	43
6	100	100	100	100	100
8	100	0	–17	–12	–
200ppm					
5	100	100	100	100	100
6	100	100	100	100	100
8	100	100	83	60	17

junction brings about a relief of the interaction between the C-4 methyl group and C-6. It is possible that the isomerisation of the ring junction takes place by enolisation of the 1-ketone and then the microbial reduction of the ketone traps this C-10 isomer. Incubation of the 1-ketone **5** also gave the known[12] 1-oxo-10β,11-dihydroxy-4α,5α,7β-eremophilane **10**. However incubation of the 2α-hydroxy-10β,11-epoxy-4α,5α,7β-eremophilane **8** only gave the acetate ester.

In conclusion, we have examined the inhibitory action of some eremophilane analogues of sesquiterpenoid phytoalexins against the plant pathogenic fungus, *B.cinerea*. We have shown that 1α,11-dihydroxy-4α,5α,7β,10β(H)-eremophilane **6** possesses sufficient inhibitory activity to suggest that it might make a suitable lead for further investigation.

Experimental

General experimental details: Silica for chromatography was Merck 9385. Light petroleum refers to the fraction b.p. 60–80°C. ^{1}H and ^{13}C NMR spectra (see Table 2) were determined at 300 and 75 MHz respectively for solutions in deuteriochloroform. IR spectra were determined as nujol mulls. Mass spectra were determined on a Fisons Autospec mass spectrometer. Extracts were dried over sodium sulfate. The eremophilanes **4**–**7** were prepared as described previously[10,12] except that the oxymercuration of valencene was carried out for 72 h at 35°C.

Preparation of 10β,11-epoxy-2α-hydroxy-4α,5α,7β-eremophilane: Mercury(II) acetate (2.9 g) in tetrahydrofuran:water (1:1)(18 cm^3) was added to a stirred solution of 2α-hydroxy-4α,5α,7β-eremophila-1(10),11-diene[11]**7** (1 g) in tetrahydrofuran:water (1:1)(10 cm^3). The mixture was stirred for 48 h at 35°C. Sodium hydroxide (3M, 14 cm^3) and a solution of sodium borohydride (0.26 g) in sodium hydroxide (3M, 14 cm^3) were added and the mixture was stirred at room temperature for 24 h. The organic phase was extracted with ether and the extract was washed with water, brine and dried. The solvent was evaporated and the residue chromatographed on silica. Elution with 15% ethyl acetate:light petroleum gave 10β,11-epoxy-2α-hydroxy-4α,5α,7β-eremophilane **8a** (500 mg) which crystallised from ethyl acetate as needles, m.p. 80–82°C, (Found: M$^+$ 238.1934 $C_{15}H_{26}O_2$ requires 238.1933), ν_{max}/cm^{-1} 3386; δ_H 0.86 (3H, d, *J*=7.0 Hz,), 0.88 (3H, s,), 1.19 (3H, s), 1.27 (3H, s), 3.99(1H, tt, *J*=4.8, 11.4 Hz).

General fermentation conditions: *Botrytis cinerea* (UCA 992) was grown on shake culture at 25°C in 250 cm^3 conical flasks each containing medium (75 cm^3) comprising (per litre), glucose (40 g), yeast extract (1 g), potassium dihydrogen phosphate (5 g), sodium nitrate (2 g), magnesium sulfate (0.5 g), iron(II) sulfate 10 mg) and zinc sulfate (5 mg). The fermentations were grown for 3 d. before the substrates in ethanol (1 cm^3 per flask) were added. After a further 7 d the mycelium was filtered, washed with water and ethyl acetate. Sodium chloride was added to the broth and it was acidified to pH 2. The broth was extracted with ethyl acetate and the extract was combined with the ethyl acetate washings of the mycelium. The combined extracts were washed with aqueous sodium hydrogen carbonate, water, brine and dried. The solvent was evaporated and the fermentation products were chromatographed on silica.

Table 2 ^{13}C NMR data for eremophilanes

Carbon atom	Compound **5**	**6**	**8a**	**9**	**10**
1	213.1	72.8	42.3	70.5	213.4
2	21.4	25.7	66.6	17.2	36.6
3	33.0	26.6	39.0	28.9	30.7
4	42.0	43.2	36.0	40.6	33.4
5	41.7	36.2	37.5	36.3	42.9
6	41.2	40.9	37.4	39.0	31.7
7	43.0	43.4	35.1	38.3	42.0
8	26.8	25.5	21.5	24.5	20.7
9	39.2	39.1	29.3	30.7	29.5
10	57.7	48.9	77.7	44.5	78.0
11	72.7	71.7	73.9	72.8	72.7
12	27.4	26.6	28.3	20.3	27.0
13	28.2	27,5	29.4	27.8	27.2
14	14,5	15.0	19.7	20.3	14.1
15	12.0	14.5	15.7	15.6	12.8

The metabolites were eluted with mixtures of ethyl acetate and light petroleum.

(a) 1α,11-Dihydroxy-4α,5α,7β,10β(H)-eremophilane **6** (500 mg) in ethanol (16 cm^3) was evenly distributed between 16 flasks. Chromatography of the fermentation products obtained as above, gave, in the fraction eluted with 20% ethyl acetate:light petroleum, 11-hydroxy-1-oxo-4α, 5α, 7β,10β(H)-eremophilane **5** (20 mg) identified by its ^{1}H NMR spectra. Further elution with 25% ethyl acetate:light petroleum gave 1β,11-dihydroxy-4α,5α,7β,10α(H)-eremophilane **9** (15 mg) which crystallised from ethyl acetate as needles, m.p. 78–80°C, (Found: M$^+$ 240.2089 $C_{15}H_{28}O_2$ requires 240.2089), ν_{max}/cm^{-1} 3331; δ_H=0.85 (3H, d, *J*=6.8 Hz,), 0.89 (3H, s,), 1.14 (3H, s), 1.17 (3H, s), 1.34 (1H, td, *J*=3.7,11.3 Hz), 3.90 (1H, dt, *J*=11.3, 4.6 Hz).

(b) 11-Hydroxy-1-oxo-4α,5α,7β,10β(H)-eremophilane **5** (520 mg) in ethanol (16 cm^3) was evenly distributed between 16 flasks. The fermentation products were chromatographed on silica. Elution with 25% ethyl acetate:light petroleum gave 10β,11-dihydroxy-1-oxo-4α,5α,7β-eremophilane **10** (20 mg), m.p. 179–181°C (lit.,[12] 182–183°C) identified by its IR and ^{1}H NMR spectra.

(c) 10β,11-Epoxy-2α-hydroxy-4α,5α,7β-eremophilane **8a** (210 mg) in ethanol (16 cm^3) was evenly distributed between 16 flasks. The fermentaion products were chromatographed on silica. Elution with 5% ethyl acetate:light petroleum gave 2α-acetoxy-10β,11-epoxy-4α,5α,7β-eremophilane **8b** (10 mg) as an oil, (Found: M$^+$ 280.2021 $C_{17}H_{28}O_3$ requires 280.2038), ν_{max}/cm^{-1} 1743; δ_H=0.83 (3H, d, *J*=7.0 Hz,), 0.86 (3H, s,), 1.17 (3H, s), 1.24 (3H, s), 1.98 (3H, s), 5.02 (1H, tt, *J*=11.5, 4.6 Hz).

X-Ray crystal data and structure determinations: (a) 1α,11-dihydroxy-4α,5α,7β,10β(H)-eremophilane **6**, $C_{15}H_{28}O_2$, M_r 240.37, monoclinic, space group C2 (No.5), a = 27.055(2), b = 15.1011(7), c = 12.5902(10)Å, α = γ = 90°, β = 114.947(3)°, V = 4664.0(6)Å^3, Z = 12, D_{calc} 1.03 g/cm^3, μ = 0.07 mm^{-1}, F(OOO) = 1608, crystal size = 0.40 × 0.30 × 0.01 mm^3. A total of 14124 reflections were collected on a KappCCD diffractometer for 3.71 < θ < 25.05° and –32 ≤ h ≤ 28, –15 ≤ k ≤ 17, –15 ≤ l ≤ 11. There were 7893 independent reflections and 6061 reflections with $I > 2\sigma(I)$ were used in the refinement. The structure was solved by direct methods and refined using SHELXL-97. The final R indices were [$I > 2\ \sigma\ (I)$] R_1 = 0.072, wR_2 = 0.179 and (all data) R_1 = 0.100, wR_2 = 0.199. The largest difference peak and hole was 0.53 and –0.23 e Å^3. There were three independent molecules with the same geometry in the unit cell.

(b) 1β,11-dihydroxy-4α,5α,7β,10α(H)-eremophilane **9**, $C_{15}H_{28}O_2$, M_r 240.37, orthorhombic, space group $P2_12_12_1$ (No.19), a = 9.7942(3), b = 13.9929(8), c = 21.3562(14)Å, α = β = γ = 90°, V = 2926.9(3)Å^3, Z = 8, D_{calc} = 1.09 g/cm^3, μ = 0.07 mm^{-1}, F(OOO) = 1072, crystal size = 0.4 × 0.2 × 0.1 mm^3. A total of 12661 reflections were collected on a KappCCD diffractometer for 3.82 < θ < 25.04° and –11 ≤ h ≤ 9, –16 ≤ k ≤ 14, –25 ≤ l ≤ 20. There were 5102 independent reflections and 3602 reflections with $I > 2\sigma(I)$ were used in the refinement. The structure was solved by direct methods and refined using SHELXL-97. The final R indices were [$I > 2\sigma(I)$] R_1 = 0.057, wR_2 = 0.110 and (all data) R_1 = 0.094, wR_2 = 0.125. The largest difference peak and hole was 0.18 and –0.18 e Å^3. There were two independent molecules with the same geometry in the

unit cell. The data are deposited with the Cambridge Crystallographic Data Centre (CCDC 239172-3).

Received 18 May 2004; accepted 14 June 2004
Paper 04/2533

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