Biotransformation of the fungistatic sesquiterpenoid ginsenol by *Botrytis cinerea*

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The antifungal properties of ginsenol (**1**) have been determined. The biotransformation of this fungistatic sesquiterpenoid by the plant pathogen *Botrytis cinerea* gave the 8- and 9-hydroxy derivatives (**5** and **3**) as the major metabolites and the 6α- and 10β-hydroxy (**8** and **7**) and 8- and 9-oxo derivatives (**6** and **4**) as minor metabolites. The structure and stereochemistry of the major compound **3** were established by X-ray crystallography.

Introduction

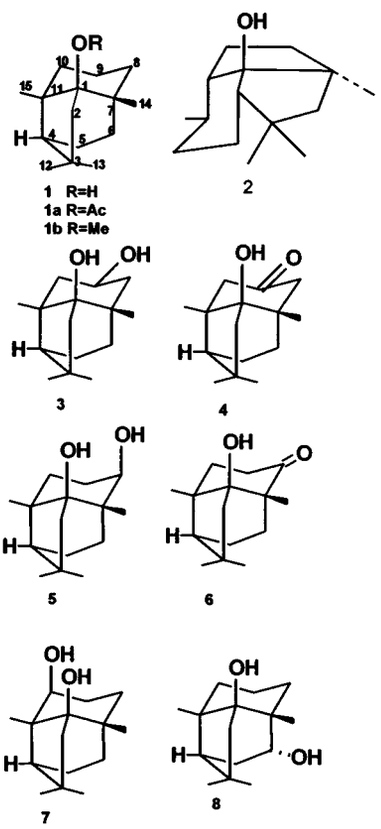
Botrytis species are serious pathogens, which are implicated in many diseases of flowers, fruits and vegetables. In particular *Botrytis cinerea* attacks economically important crops such as lettuces, carrots, tobacco, strawberries and grapes.[1] The recent development of tolerance to commercial fungicides by *B. cinerea* has led to an increase in the quantities of these compounds that have been used with the consequent additional problems of persistence and serious economic damage arising from the decreased quality of wines produced from treated grapes.[2]

Over the last few years we have undertaken a research programme directed toward the rational design of fungicides for *Botrytis* infections of commercial crops based on biosynthetic principles. Botrydial and structurally related compounds are characteristic metabolites of *Botrytis* spp. Botrydial is a bicyclic non-isoprenoid sesquiterpene, which was isolated from cultures of *B. cinerea*.[3] Its biosynthesis has been investigated.[4] Although the role of these metabolites in the fungal physiology is unknown, our results have shown that these metabolites are responsible for the typical necrotic lesions of the fungal infection and they have an important role in the expression of the phytotoxicity of the organism and its subsequent development.[5]

We are exploring the inhibition of the biosynthesis of these key secondary metabolites by analogues of botrydial precursors in order to develop a rational means of controlling the fungus and its pathogenicity. In the course of these studies we have tested the fungicidal activity of different sesquiterpenoid natural products. As a result of these studies, ginsenol **1**, a natural product isolated from *Panax ginseng*,[6] and which we have obtained by the rearrangement of isocaryophyllene,[7] displayed antifungal activity against *B. cinerea*. The aim of the work described in this paper, was to study the biotransformation of ginsenol **1** by *B. cinerea* as a part of the fungal detoxification mechanism.

Results and discussion

Ginsenol **1** is a sesquiterpenoid alcohol which is isomeric with a proposed intermediate **2** in the biosynthesis of botrydial. Its carbon skeleton is related to that of an intermediate in the formation of neoclovene.[7] The antifungal properties of **1** against *B. cinerea* were determined using the "poisoned food technique".[7] The commercial fungicide Euparen® was used as standard in this test. As shown in Fig. 1, ginsenol **1** was active above 60 ppm and gave total inhibition at 180 ppm for 4 days.



It is worth noting that the acetyl and *O*-methyl derivatives (**1a** and **1b**) were inactive indicating that the hydroxy group plays an important role in the inhibitory mechanism. However the inhibitory effect of ginsenol **1** diminished with time suggesting that the fungus possessed a detoxification mechanism.

In order to identify the compounds that are produced by this detoxification pathway, an ethanolic solution of ginsenol **1** was evenly distributed between 10 Roux bottles of *B. cinerea* at a concentration of 150 ppm and the surface culture was then incubated for a further 6 days. The fermentation was then extracted with ethyl acetate and the metabolites were separated by chromatography on silica. In addition to the starting material, the following six new metabolites were isolated: 9β-hydroxyginsenol **3**, 9-oxoginsenol **4**, 8β-hydroxyginsenol **5**,

Table 1 ^{13}C NMR data of compounds **1**, **3**–**8** (100 MHz, $CDCl_3$)

C	1[6]	3	4	5	6	7	8
1	83.1°	82.4°	82.3°	85.3°	—	84.9°	82.3°
2	48.4$^-$	47.8$^-$	48.2$^-$	49.7$^-$	49.3$^-$	49.0$^-$	47.8$^-$
3	35.5°	35.6°	36.1°	35.5°	35.9°	35.8°	36.0°
4	57.4$^+$	56.0$^+$	56.1$^+$	56.8$^+$	56.0$^+$	52.7$^+$	55.3$^+$
5	26.4$^-$	26.6$^-$	23.9$^-$	26.5$^-$	24.4$^-$	25.6$^-$	27.4$^-$
6	34.6$^-$	34.3$^-$	34.3$^-$	30.9^{-a}	32.8$^-$	34.0^{-a}	74.2$^+$
7	38.4°	40.2°	37.2°	43.6°^{b}	53.1°	38.3°	42.9°
8	34.7$^-$	44.7	51.1$^-$	72.0$^+$	213.1°	34.6^{-a}	35.4$^-$
9	22.0$^-$	66.6$^-$	207.0°	25.9$^-$	36.9$^-$	31.4$^-$	21.4$^-$
10	33.7$^-$	44.2$^-$	50.7$^-$	32.2^{-a}	31.9$^-$	79.1$^+$	33.6$^-$
11	45.5°	48.8°	43.4°	44.8°^{b}	45.0°	50.4°	45.0°
12	34.5$^+$	34.3$^+$	34.5$^+$	34.3$^+$	34.2$^+$	34.1$^+$	34.3$^+$
13	28.6$^+$	28.6$^+$	27.7$^+$	28.6$^+$	28.7$^+$	28.6$^+$	29.0$^+$
14	24.7$^+$	24.5$^+$	24.0$^+$	19.4$^+$	17.6$^+$	24.2$^+$	21.1$^+$
15	29.1$^+$	28.8$^+$	27.9$^+$	28.7$^+$	28.1$^+$	27.2$^+$	29.2$^+$

Amplitude of signals in DEPT spectrum (Me or CH = +, CH_2 = −; quaternary C = o). [a,b] Assignments may be interchanged.

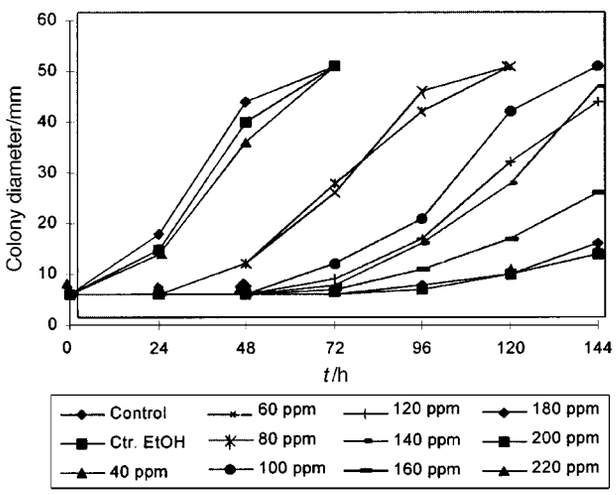


Fig. 1 Inhibition assay for compound **1**.

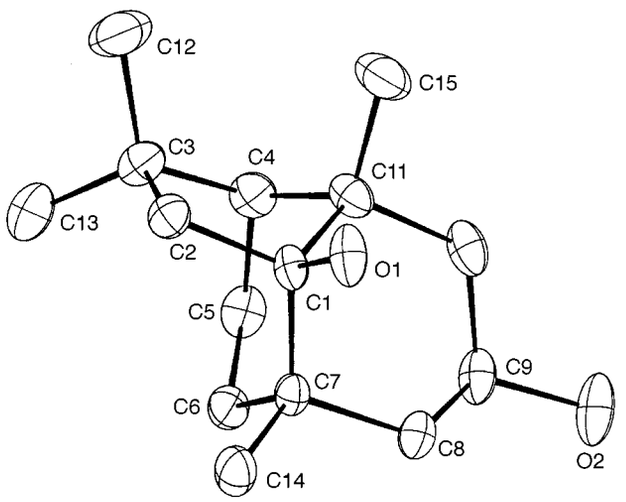


Fig. 2 ORTEP drawing of **3**.

8-oxoginsenol **6**, 10β-hydroxyginsenol **7** and 6α-hydroxyginsenol **8**.

The ^{13}C NMR and mass spectra of compound **3** were consistent with the molecular formula, $C_{15}H_{26}O_2$, whilst the IR spectrum contained a hydroxy absorption. The ^1H and ^{13}C NMR spectra were very similar to those of ginsenol **1** except for the presence of signals at δ_H 4.23 and δ_C 66.6 and the downfield shift of two methylene signals (see Table 1). Comparison of the spectra with those of ginsenol **1** indicated that the compound was a 9-hydroxy derivative. Its structure and stereochemistry were established by X-ray crystallography (see Fig. 2).

Compound **4** showed IR absorption at 1696 cm^{-1} and a ^{13}C NMR signal at δ_C 207.0 consistent with the presence of a ketone. Many of the signals in the ^1H and ^{13}C NMR spectra were similar to those of **3**. However the two methylene signals assigned to C(8) and C(10) were further deshielded indicating that the 9-hydroxy group had been oxidized to a ketone.

The spectroscopic data of alcohol **5** (ν_{max} 3447 cm^{-1}, δ_C 72.0) were also similar to those of **3**. However there was a downfield shift for the ^{13}C NMR signals assigned to C(7) and C(9) and a γ-gauche shielding of the signal corresponding to C(14) in agreement with the structure of 8-hydroxyginsenol for **6**. The β-stereochemistry for the hydroxy group at C(8) followed from the nuclear Overhauser enhancement which was observed for the signal corresponding to H(14) (2%), and for both the signals assigned to H(9) (2%), when the signal (δ_H 3.95), assigned to H(8), was irradiated. A comparison of the spectroscopic data of compounds **5** and **6**, which showed a similar relationship to that observed for **3** and **4**, indicated that **6** was the ketone (ν_{max} 1701 cm^{-1}, δ_C 213.1) corresponding to the alcohol **5**. In particular the signals assigned to C(7) and C(9) showed a further downfield shift.

The IR and ^{13}C NMR spectra of compound **7** and **8** indicated the presence of a secondary alcohol in both metabolites. The downfield shift of the ^{13}C NMR signals assigned to C(9) and C(11) and the γ-*gauche* shielding of C(15) led to location of the hydroxy group at C(10) in compound **7**. The β-stereochemistry was assigned to the hydroxy group as a consequence of the nuclear Overhauser enhancements which were observed for the ^1H NMR signals corresponding to H(15) (4%) and H(4) (2%) when H(10) (δ_H 3.77) was irradiated. These enhancements are only possible if the hydroxy group has the β stereochemistry.

The location of the hydroxy group at C(6) in compound **8** followed from the downfield shift, compared to ginsenol (**1**), of the ^{13}C NMR signals assigned to C(5) and C(7) from δ_C 26.3 and 38.4 to δ_C 27.4 and 42.9 respectively. NOE experiments revealed an enhancement of the signals corresponding to H(14) (4%) and H(13) (8%) and H(2) (6%) when H(6) (δ_H 3.80) was irradiated, confirming an α-configuration for the hydroxy group.

In the course of this work we observed some changes in the growth of *B. cinerea*. Firstly the growth of the fungus was inhibited when the substrate was added to the broth. Secondly a careful examination of the broth extracts after the biotransformation showed that botrydial and its derivatives were not produced during the period in which the substrate was present in the broth. The acetyl and *O*-methyl ginsenol derivatives, **1a** and **1b**, were both inactive, and hence the tertiary hydroxy group in ginsenol **1** is important for the expression of its

biological activity. The positions that were hydroxylated were on carbon atoms that were 1,3 or 1,4 from the initial hydroxy group of ginsenol **1**. If the substrate is viewed as in **1** then it is apparent that the centres that are oxidized are those that are related to the centres involved in the biosynthetic cleavage of ring C of the botrydial precursor **2**. The poor recovery of the products from the biotransformation may be related to the further biodegradation of these compounds by Baeyer–Villiger oxidation and further fragmentation of the ring system. These results give an indication of the type of structural modifications which may be necessary if substrates of this type are to be further developed as selective fungal control agents for *B. cinerea*.

Experimental

General experimental procedures

Melting points were measured with a Reicher-Jung Kofler apparatus and are uncorrected. Optical rotations were determined with a Perkin-Elmer 241 polarimeter. $[\alpha]_D$ Values are given in 10 deg cm^{-2} mg^{-1}. IR spectra were recorded on a Perkin-Elmer 881 spectrophotometer. ^1H and ^{13}C NMR measurements were obtained on Varian Gemini 200 and Varian Unity 400 NMR spectrometers with $SiMe_4$ as internal reference. *J* Values are given in Hz. Mass spectra were recorded using a VG 12-250 and a VG Autospec spectrometer at 70 eV. HPLC was performed with a Hitachi/Merck L-6270 apparatus equipped with a UV–VIS detector (L 4250) and a differential refractometer detector (RI-71). TLC was performed on Merck Kiesegel 60 F_{254}, 0.2 mm thick. Silica gel (Merck 9385) was used for column chromatography. Purification by HPLC was accomplished using a Si gel column (Hibar 60, 7 μm, 1 cm wide, 25 cm long).

Micro-organism and antifungal assays

The culture of *Botrytis cinerea* employed in this work, *B. cinerea* (UCA 992), was obtained from grapes of Domecq vineyard, Jerez de la Frontera, Cádiz, Spain. This culture of *B. cinerea* is deposited in the Universidad de Cadiz, Facultad de Ciencias, Mycological Herbarium Collection (UCA). Bioassays were performed by measuring the inhibition of the radical growth on an agar medium in a Petri dish. The test compound was dissolved in ethanol to give a final compound concentration of 50 to 200 mg l^{-1}. Solutions of the test compound were added to glucose-malt-peptone-agar medium (61 g of glucose-malt-peptone-agar per litre, pH 6.5–7.0). The final ethanol concentration was identical in the control and treated cultures. The medium was poured into 6 cm diameter sterile plastic Petri dishes and a 5 mm diameter mycelial disc of *B. cinerea* cut from an actively growing culture was placed in the centre of the agar plate. Inhibition of radial growth was measured for six days.

Biotransformation experiment

Botrytis cinerea (UCA 992) was grown on surface culture in Roux bottles at 25 °C for 3 days on a Czapek–Dox medium (150 ml per flask) comprising (per litre of distilled water), glucose (40 g), yeast extract (1 g), potassium dihydrogen phosphate (5 g), sodium nitrate (2 g), magnesium sulfate (0.5 g), ferrous sulfate (10 mg) and zinc sulfate (5 mg). The substrate (ginsenol **1**, 150 ppm per bottle) dissolved in ethanol was distributed over 10 Roux bottles after two days growth. The fermentation was continued for a further period of 6 days. The mycelium was filtered and washed with brine and ethyl acetate. The broth was saturated with sodium chloride, acidified (pH 2) and extracted with ethyl acetate. The extracts were separated into acidic and neutral fractions with aqueous sodium hydrogen carbonate. The acid fraction was recovered in ethyl acetate. The extracts were dried over sodium sulfate, the solvent was evaporated and the residues were chromatographed on a gel column and then by HPLC. From the neutral fractions, the following compounds were isolated: ginsenol (**1**, 69 mg), 9β-hydroxyginsenol (**3**, 25 mg), 9-oxoginsenol (**4**, 5 mg), 8β-hydroxyginsenol (**5**, 25 mg), 8-oxoginsenol (**6**, 1.5 mg), 10β-hydroxyginsenol (**7**, 7 mg), 6α-hydroxyginsenol (**8**, 7.5 mg).

9β-Hydroxyginsenol 3. Isolated as a colourless solid; mp 138–140 °C; $[\alpha]_D$ −28 (*c* 1 in $CHCl_3$); ν_{max}(film)/cm^{-1} 3382, 1423, 1023; δ_H (400 MHz; $CDCl_3$) 0.90 (3H, s, 14-H), 1.03 (3H, s, 13-H), 1.23 (1H, dd, $J_{8\beta\text{-}8\alpha}$ 14.6, $J_{8\beta\text{-}9}$ 10.0, 8β-H), 1.26 (3H, s, 15-H), 1.27 (3H, s, 12-H), 1.35 (1H, m, 4-H), 1.68 (1H, d, $J_{2\alpha\text{-}2\beta}$ 14.2, 2α-H), 1.99 (1H, d, $J_{2\beta\text{-}2\alpha}$ 14.2, 2β-H), 2.20 (1H, br dd, $J_{8\alpha\text{-}8\beta}$ 14.6, $J_{8\alpha\text{-}9}$ 6.8, 8α-H), 4.23 (1H, m, 9-H); ^{13}C NMR data, Table 1; *m*/*z* (int. rel.) 238 (M^+, 0.1%), 220.1827 ($M^+ - 18$, 17%. $C_{15}H_{24}O$ requires 220.1827), 205 (63), 178 (29), 162 (33), 151 (28), 138 (61), 41 (100).

9-Oxoginsenol 4. Obtained as a colourless oil; $[\alpha]_D$ −32 (*c* 1 in $CHCl_3$); ν_{max}(film)/cm^{-1} 3465, 1696, 1288, 1036; δ_H (400 MHz; $CDCl_3$) 0.97 (3H, s, 14-H), 1.07 (3H, s, 13-H), 1.31 (3H, s, 12-H), 1.38 (3H, s, 15-H), 1.39 (1H, m, 4-H), 1.49 (2H, m, 6α-H and 5-H), 1.66 (2H, m, 6β-H and 5-H′), 1.77 (1H, d, $J_{2\beta\text{-}2\alpha}$ 14.2, 2β-H), 1.86 (1H, dd, $J_{8\beta\text{-}8\alpha}$ 13.9, $J_{8\beta\text{-}10\beta}$ 2.4, 8β-H), 2.06 (1H, d, $J_{2\alpha\text{-}2\beta}$ 14.2, 2α-H), 2.14 (1H, d, $J_{10\alpha\text{-}10\beta}$ 17.1, 10α-H), 2.54 (1H, dd, $J_{10\beta 10\alpha}$ 17.1, $J_{10\beta\text{-}8\beta}$ 2.4, 10β-H), 2.83 (1H, dq, $J_{8\alpha\text{-}8\beta}$ 13.9, 8α-H); ^{13}C NMR data, Table 1; *m*/*z* (int. rel.) 236.1780 (M^+, 23%. $C_{15}H_{24}O_2$ requires 236.1776), 221 ($M^+ - 15$, 30), 203 (69), 175 (52), 167 (49), 135 (56), 95 (100).

8β-Hydroxyginsenol 5. Obtained as a colourless solid; mp 123–125 °C; $[\alpha]_D$ −16 (*c* 1 in $CHCl_3$); ν_{max}(film)/cm^{-1} 3447, 1373, 736; δ_H (400 MHz, $CDCl_3$) 0.96 (3H, s, 14-H), 1.05 (3H, s, 13-H), 1.18 (3H, s, 15-H), 1.26 (3H, s, 12-H), 1.53 (1H, m, 4-H), 1.65 (1H, d, $J_{2\beta\text{-}2\alpha}$ 14.2, 2β-H), 1.83 (1H, m, 9-H), 2.05 (1H, d, $J_{2\alpha\text{-}2\beta}$ 14.2, 2α-H), 3.95 (1H, br s, 8-H); ^{13}C NMR data, Table 1; *m*/*z* (int. rel.) 238.1939 (M^+, 2%. $C_{15}H_{26}O_2$ requires 238.1933), 220 ($M^+ - 18$, 48), 205 ($M^+ - 18 - 15$, 100), 149 (27), 138 (58), 121 (60).

8-Oxoginsenol 6. Obtained as a colourless oil; $[\alpha]_D$ +4 (*c* 0.5 in $CHCl_3$); ν_{max}(film)/cm^{-1} 3371, 1701; δ_H (400 MHz, $CDCl_3$) 1.07 (3H, s, 14-H), 1.11 (3H, s, 13-H), 1.31 (3H, s, 12-H), 1.32 (3H, s, 15-H), 1.55 (1H, m, 4-H), 1.73 (1H, d, $J_{2\beta\text{-}2\alpha}$ 14.2, 2β-H), 1.99 (1H, d, $J_{2\alpha\text{-}2\beta}$ 14.2, 2α-H), 2.18 (1H, ddd, $J_{10\beta\text{-}10\alpha}$ 14.9, $J_{10\beta\text{-}9\alpha}$ 8.3, $J_{10\beta\text{-}9\beta}$ 6.1, 10β-H), 2.48 (1H, ddd, $J_{9\beta\text{-}9\alpha}$ 15.7, $J_{9\beta\text{-}10}$ 8.3, $J_{9\beta\text{-}10'}$ 8.3, 9β-H), 2.57 (1H, ddd, $J_{9\alpha\text{-}9\beta}$ 15.7, $J_{9\alpha\text{-}10\beta}$ 8.8, $J_{9\alpha\text{-}10}$ 6.1, 9α-H); ^{13}C NMR data, Table 1; *m*/*z* (int. rel.) 236.1658 (M^+, 27%. $C_{15}H_{24}O_2$ requires 236.1776), 221 ($M^+ - 15$, 24), 203 ($M^+ - 18 - 15$, 9), 180 (88), 151 (90), 125 (56).

10β-Hydroxyginsenol 7. Obtained as a colourless oil; $[\alpha]_D$ −8 (*c* 1 in $CHCl_3$); ν_{max}(film)/cm^{-1} 3444, 997; δ_H (400 MHz, $CDCl_3$) 0.86 (3H, s, 14-H), 1.04 (3H, s, 13-H), 1.27 (3H, s, 12-H), 1.42 (3H, s, 15-H), 1.57 (1H, m, 4-H), 1.68 (1H, d, $J_{2\beta\text{-}2\alpha}$ 14.1, 2β-H), 1.89 (2H, m, 9-H), 1.97 (1H, d, $J_{2\alpha\text{-}2\beta}$ 14.1, 2α-H), 3.77 (1H, dd, $J_{10\text{-}9}$ 8.5, $J_{10\text{-}9'}$ 9.9, 10-H); ^{13}C NMR data, Table 1; *m*/*z* (int. rel.) 238.1938 (M^+, 5%. $C_{15}H_{26}O_2$ requires 238.1933), 223 (20), 220 (4), 205 (10), 202 (1), 149 (11), 138 (25), 125 (100), 123 (42).

6α-Hydroxyginsenol 8. Obtained as a white solid, mp 33–35 °C; $[\alpha]_D$ −24 (*c* 1 in $CHCl_3$); ν_{max}(film)/cm^{-1} 3435, 1011; δ_H (400 MHz, $CDCl_3$) 0.96 (3H, s, 14-H), 1.06 (3H, s, 13-H), 1.21 (3H, s, 15-H), 1.28 (3H, s, 12-H), 1.68 (1H, d, $J_{2\beta\text{-}2\alpha}$ 14.4, 2β-H), 1.88 (1H, m, 5-H), 1.91 (1H, d, $J_{2\alpha\text{-}2\beta}$ 14.4, 2α-H), 1.99 (1H, m, 5′-H), 3.80 (1H, m, 6-H); ^{13}C NMR data, Table 1; *m*/*z* (int. rel.) 238.1917 (M^+, 17%. $C_{15}H_{26}O_2$ requires 238.1933), 223 (30), 220 (3), 205 (13), 202 (2), 177 (10), 149 (11), 125 (42), 43 (100).

Structure determination for compound 3

Crystal data. $C_{15}H_{26}O_2$, $M = 238.4$. Crystal system orthorhombic; $a = 7.072(4)$, $b = 11.201(2)$, $c = 17.660(4)$ Å, $\alpha = \beta = \gamma = 90°$; $V = 1898.9$ Å^3, $0.40 \times 0.20 \times 0.20$ mm, temperature 293(2) K, space group $P2_12_12_1$ (No. 19); $Z = 4$, $\lambda = 0.71071$ Å, $D_x = 1.13$ mg m^{-3}; colourless, $F(000) = 528$, $\mu = 0.07$ mm^{-1}.

Data collection and processing. 1946 reflections collected, 1481 with $I < 2\sigma(I)$.

Structure solution and refinement. Automatic direct method. Full-matrix least-squares on all F^2 with all non hydrogen atoms anisotropic. Hydrogen atoms were included in riding mode with $U_{iso}(H) = 1.2U_{eq}(C)$ or 1.5 $U_{eq}(C)$ for methyl groups. Hydroxy groups were fixed at idealised geometry but with the torsion angle defining the hydrogen atom position refined and $U_{iso}(H) = 1.5U_{eq}(O)$. Final R_1 indices $[I < 2\sigma(I)]$ 0.055, $wR_2 = 0.131$. R indices (all data) $R_1 = 0.075$, $wR_2 = 0.146$. S$[F^2]$ 1.029 for 157 refined parameters. The largest remaining electron density peak was 0.20 e Å^{-3}. Maximum shift/e.s.d. 0.006. Programs used: data collection Enraf-Nonius CAD4 software;[8] structure solution SHELXS-86;[9] structure refinement SHELXL-93;[10] interactive graphics and final drawing CAMERON.[11] Full crystallographic details, excluding structure factor tables, have been deposited at the Cambridge Crystallographic Data Centre (CCDC). For details of the deposition scheme, see 'Instructions for Authors', *J. Chem. Soc.*, *Perkin Trans. 1*, available *via* the RSC Web page (http://www.rsc.org/authors). Any request to the CCDC for this material should quote the full literature citation and the reference number 207/298.

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