Secondary Mould Metabolites. Part 47.[1] Isolation and Structure Elucidation of Clavilactones A–C, New Metabolites from the Fungus *Clitocybe clavipes*

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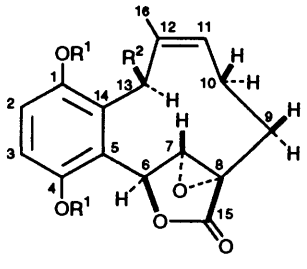
Clavilactones A–C (**1**, **4** and **5**) have been isolated from cultures of the Basidiomycetous fungus *Clitocybe clavipes*. Their structures and relative configurations have been deduced from ^1H and ^{13}C NMR studies, chemical reactions and single-crystal X-ray analysis of the dimethyl ether derivative **3**. ^1H–^1H coupling constants, NOE data and molecular modelling calculations all suggest that the molecules have little conformational mobility and the conformation adopted in solution by clavilactone A **1** and dimethyl ether derivative **3** is essentially identical with that of **3** in the solid state. Clavilactones A–C exhibit antifungal and antibacterial activity and inhibit the growth germination of *Lepidium sativum*. A possible biogenetic origin of clavilactone A **1** is discussed.

During the screening of the genus *Clitocybe* (*Basidiomycetae*) for active secondary metabolites, we have reported the isolation of a large number of new compounds,[2–7] principally protoilludanic sesquiterpenes. In the present study, a culture of *C. clavipes*, a non-toxigenic fungus whose fruit-body is named *Agaricus clavatus*, was investigated. Extraction of a pure culture grown in a solid medium (malt–peptone–glucose–agar) with ethyl acetate, followed by flash chromatography on silica gel, led to the isolation of three main metabolites, designated clavilactone A **1**, B **4** and C **5**.



1 $R^1 = R^2 = H$
2 $R^1 = Ac$, $R^2 = H$
3 $R^1 = Me$, $R^2 = H$
4 1,4-dioxo, $R^2 = H$
5 $R^1 = H$, $R^2 = OH$

Clavilactone A, $C_{16}H_{16}O_5$, showed IR bands at 3400 (OH) and 1750 (CO) cm^{-1} and formed a diacetate **2** and a dimethyl ether. The structure of the latter was shown by X-ray crystallography to be **3** and therefore clavilactone A is deduced to have structure **1**.

The broad-band ^1H-decoupled ^{13}C NMR spectrum of **1** (Table 1) contained 16 signals. The sp^2 signals were assigned to one ester-like carbonyl carbon (C-15) and to the carbons of a tetrasubstituted aromatic ring and of a trisubstituted double bond. The sp^3 signals were assigned to one methyl, three methylene and three oxygen-bearing carbons. Two of them, C-7 and C-8, were attributed to a trisubstituted oxirane ring on the basis of the ^1J(C, H) of 198 Hz exhibited by C-7, while C-6, which showed a coupling of ^3J(C, H) of 2.0 Hz with C-15, was linked to an ester-like oxygen as depicted in Fig. 1.

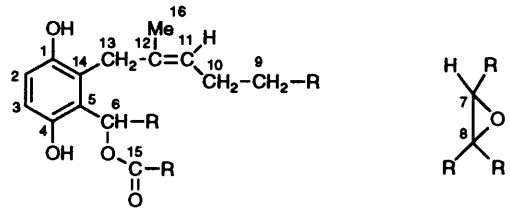


Fig. 1 Part structure of compound **1**

A detailed analysis of the ^1H NMR spectrum of compound **1** (Table 2) confirmed the above results indicating that the aromatic protons are *ortho*-coupled and pointed to the presence of the R–C(13)H_2–C(12)(Me)=C(11)H–C(10)H_2–C(9)H_2R grouping. Finally, the cross-peaks observed in a COLOC spectrum optimized for the observation of two- and three-bond ^1H–^{13}C couplings of ~6 Hz between 6-H and the quaternary C-4, C-5 and C-14 carbons and between 13-H_2 and the quaternary C-1, C-5 and C-14 carbons indicated that C-6 and C-13 are located at C-5 and C-14, respectively, and, as a consequence, that the two hydroxy groups are *para*-disposed.

The (C, H) couplings of 8.0 and 2.0 Hz observed between 9-H_2 and C-15 suggest that these atoms are coupled *via* three- and not four-bond interactions. It follows that both C-9 and C-15 must be connected to C-8 and hence C-6 to C-7. It must be noted that no vicinal coupling was observed between 6- and 7-H. This could be due to the fact that these protons form a dihedral angle of ~90° provided that the relative configuration, assumed as *S* in the formula, is the same for the C-6, C-7 and C-8 carbons.

A view of only one of the two crystallographically independent molecules of **3** is presented in Fig. 2, since both adopt the same conformation and cannot be distinguished visually. The configuration displayed in Fig. 2 is arbitrary as the diffraction data did not indicate any significant preference for either enantiomer. Bond lengths are in the expected range while, for the sake of a clearer discussion, selected torsion angles are reported in Table 3.

The molecule can be described as a ten-membered ring (involving atoms from C-5 through C-14), whose conformation is constrained by condensation with a phenyl group at C-14 and

Table 1 ^{13}C NMR data for compounds **1**, **2** and **5** in [$^2H_6$]acetone

Carbon atom	1		2[a]		5	
	δ_C	^1J(C, H)/Hz	δ_C	^1J(C, H)/Hz	δ_C	^1J(C, H)/Hz
1	150.32		149.30[b]		152.50	
2	118.39	159	126.26	166.5	120.97	161
3	115.34	161	123.04	167.5	117.00	161
4	150.32		148.80[b]		150.07	
5	120.86		127.32		117.54	
6	75.90	155.5	75.80	155.5	75.64	156
7	64.28	198	63.91	200	64.63	198
8	62.08		61.82		62.04	
9	26.02	131.5	25.23	131.5	24.51	131.5
10	23.08	128.5	23.09	128.5	23.19	129
11	122.44	154	123.87	154	127.40	155
12	138.88		136.90		140.24	
13	27.99	127	28.76	127.5	69.51	141
14	127.82		133.62		126.12	
15	173.11		172.40		173.10	
16	21.61	126.5	22.00	126.5	18.53	127

[a] The carbonyl and methyl carbons of the acetate groups resonate at δ_C 169.92, 169.42 and 20.97, 20.89, respectively. [b] Assignments may be interchanged.

Table 2 ^1H NMR data for compounds **1**–**3** and **5** in [$^2H_6$]acetone

Proton	δ_H				J/Hz				
	1	2	3[a]	5	J(H, H)	1	2	3[a]	5
2	6.88	7.36[b]	6.93[b]	6.75	2,3	8.7	8.7	8.9	8.8
3	6.79	7.21[b]	6.82[b]	6.86	6,9β	1.0	0.9	1.0	1.1
6	6.30	5.80	6.38	6.32	7.9β	0.7	0.7	0.7	0.7
7	4.20	4.23	4.01	4.08	9α,9β	13.6	13.6	13.5	13.5
9α	1.29	1.31	1.27	1.29	9α,10α	3.0	3.1	3.0	2.9
9β	2.60	2.59	2.72	2.58	9α,10β	14.0	13.9	14.0	13.8
10α	2.17	2.23	2.17	2.30	9β,10α	5.0	5.0	5.0	5.1
10β	2.42	2.32	2.47	2.46	9β,10β	2.4	2.5	2.4	2.4
11	5.29	5.35	5.25	5.43	10α,10β	14.4	14.4	14.4	14.2
13α	3.02	3.14	3.01	5.49	10α,11	6.8	7.0	6.9	7.0
13β	3.70	3.43	3.72		10α,13β	1.0	1.0	1.0	
16	1.60	1.51	1.48	1.72	10α,16	0.8	0.8	0.8	0.9
1-OR	8.19	2.38[c]	3.81[c]	9.53	10β,11	10.0	10.0	10.0	10.2
4-OR	8.49	2.34[c]	3.79[c]	8.50	11,13α	0.6	0.6	0.6	0.6
13-OH				6.23	11,13β	2.8	2.7	2.8	
					11,16	1.5	1.5	1.5	1.6
					13α,13β	15.2	15.3	15.3	
					13α,16	1.4	1.4	1.4	

[a] In $CDCl_3$. [b,c] Assignments may be interchanged.

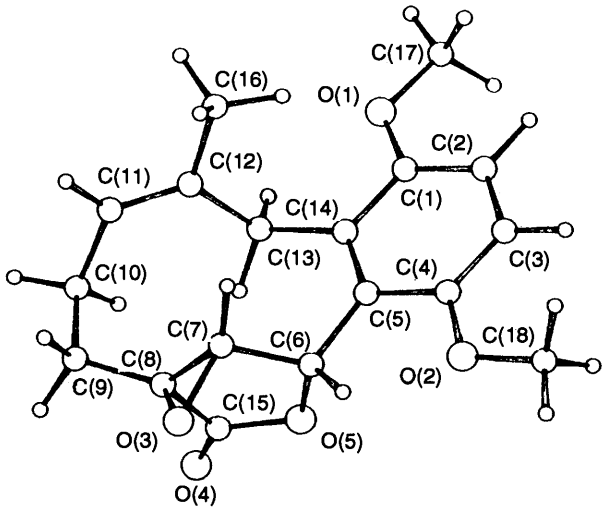


Fig. 2 A view of one of the two crystallographically independent molecules of compound **3**

C-5, and with a double bond between C-11 and C-12, requiring respectively C-10, C-11, C-12 and C-13, and C-6, C-5, C-14 and C-13 to be coplanar. Further constraints on the ten-membered ring conformation result from epoxidation at C-7, C-8 and from the rigorously planar five-membered lactone ring comprising atoms C-6, C-7, C-8, C-15 and O-5. Some bond angle deformation is apparent, specifically at the highly strained atom C-8. Short non-bonded transannular interactions[8] involving C-7 and respectively C-11 [3.27(1) Å], C-12 [3.14(1) Å] and C-13 [3.18(1) Å] also imply considerable strain. As expected on theoretical grounds[9,10] the methoxy groups deviate only marginally from the phenyl plane (see Table 3). Calculations performed on **3** with various force fields[11,12] suggest that the structure determined by X-ray diffraction is indeed very close to the minimum energy conformation for the isolated molecule as the average difference between experimental and calculated torsion angles listed in Table 3 is less than 3.4° (max 7.5°). A Monte Carlo conformational search lead to the identification of only one clearly distinct low energy conformer (disregarding the conformation of the methoxy side chains), about 8 kJ mol^{-1} less stable than the minimum energy structure.

Table 3 Selected torsion angles for the two independent molecules of **3**

Selected torsion angles	Molecule 1 (°)	Molecule 1′ (°)
C(5)–C(6)–C(7)–C(8)	121.0(6)	118.8(6)
C(6)–C(7)–C(8)–C(9)	−152.2(7)	−154.3(7)
C(7)–C(8)–C(9)–C(10)	80.0(9)	82.8(9)
C(8)–C(9)–C(10)–C(11)	−72.4(7)	−71.1(7)
C(9)–C(10)–C(11)–C(12)	96.3(8)	94.3(8)
C(10)–C(11)–C(12)–C(13)	4.5(11)	5.1(11)
C(11)–C(12)–C(13)–C(14)	−138.7(6)	−142.4(6)
C(12)–C(13)–C(14)–C(5)	88.3(7)	87.0(7)
C(13)–C(14)–C(5)–C(6)	−3.6(10)	1.2(10)
C(14)–C(5)–C(6)–C(7)	−49.8(9)	−53.2(9)
C(6)–C(7)–C(8)–C(15)	0.7(7)	1.6(7)
C(7)–C(8)–C(15)–O(5)	−0.9(8)	1.2(8)
C(8)–C(15)–O(5)–C(6)	0.7(8)	−3.6(8)
C(15)–O(5)–C(6)–C(7)	−0.2(7)	4.5(7)
O(5)–C(6)–C(7)–C(8)	−0.3(7)	−3.6(7)
C(6)–C(7)–O(3)–C(8)	−99.4(6)	−98.8(6)
C(8)–O(3)–C(8)–C(9)	−117.6(7)	−118.3(7)
C(17)–O(1)–C(1)–C(14)	−172.7(6)	−173.4(6)
C(18)–O(2)–C(4)–C(5)	171.8(6)	178.0(6)

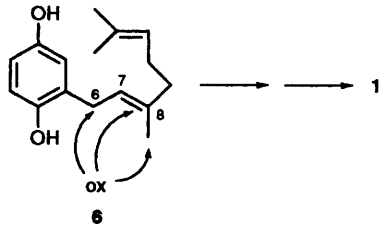
NOE experiments carried out on clavilactone A **1** (see Experimental section) indicated that the molecular conformation assumed in solution by **1**, and hence by its dimethyl ether **3** because the two compounds presented similar ^1H–^1H coupling constants (Table 2) for the ten-membered ring protons, is almost identical with that exhibited by **3** in the solid state. In fact, the NOEs observed between 7-Hβ and 9-Hα (2%), 11-H (1%), 13-Hα (1%) and 16-H_3 (0.5%) confirmed the above mentioned transannular interactions while the NOEs observed between 9-Hα and 7-Hβ (3.5%) and 11-H (2.5%) and between 10-Hβ, which is positioned *anti* with respect to 9-Hα (^3J 14.0 Hz), and 13-Hα (5%) indicated the close proximity of these protons and the little conformational mobility of the ring.

The IR spectrum of the major metabolite, clavilactone B **4**, $C_{16}H_{14}O_5$, exhibited, in addition to the band at 1760 cm^{-1} attributable to an ester-like function, a large band at 1655 cm^{-1} which suggested the presence of quinone carbonyl groups, while the ^1H NMR spectrum (see Experimental section) showed the lack of aromatic hydroxy groups. Accordingly, reduction of clavilactone B **4** with $NaBH_4$ in MeOH led to its facile conversion into clavilactone A **1** whereas reductive acetylation (Ac_2O, Zn) of **4** gave the diacetyl derivative **2**. Oxidation of clavilactone A with DDQ gave back clavilactone B.

Comparison of the ^{13}C and ^1H NMR spectra of the third metabolite, clavilactone **5**, $C_{16}H_{16}O_6$, with those of **1** (Tables 1 and 2) readily revealed the presence in **5** of a C(13)HOH fragment (δ_H 6.23 and 5.49; δ_C 69.51) instead of the C(13)H_2 group (δ_H 3.70 and 3.02; δ_C 27.99). The close similarity of the ^1H–^1H coupling constants observed in the above compounds implies that the alicyclic rings adopt an analogous preferred conformation; in particular, the value of 0.6 Hz observed for the allylic coupling between 11- and 13-H indicates that the additional hydroxy group has replaced 13-Hβ. Here again, significant NOEs were observed between 7-Hβ and 9-Hα (2%), 11-H (1%), 13-Hα (1.5%) and 16-H_3 (0.5%). It follows that the relative configuration of **5** is 6*S*,7*S*,8*S*,13*R*.

The biogenesis of clavilactones may be rationally accounted for by starting from geranylhydroquinone **6**; the conversion of **6** into **1** requires several oxidative steps, *viz.* the oxidation of the benzylic C-6 and of 8-Me, the epoxidation of the C-7–C-8 double bond and the activation of the terminal method group followed by lactonization and ring closure (Scheme 1). Several examples of such biooxidations on the geranyl chain of clavilactones can be found in the germacrane sesquiterpenoids.[13]

Clavilactone B **4** showed antibacterial activity against



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Scheme 1

Bacillus subtilis, *B. cereus*, *Sarcinea lutea* and *Saccharomyces cerevisiae* (50 μg $disc^{-1}$), while clavilactone A **1** was active only on the first at a concentration of 100 μg. All the clavilactones exhibited antifungal activity; in particular, they were active in antifungal tests performed by means of bioautography on *Cladosporium cladosporioides* and *C. cucumerinum* with amounts as low as 50 μg per plate. Compound **4** also inhibited the growth of *Lepidium sativum*.[14]

Isolation of new components from the same strain and tests on their biological activity are in progress.

Experimental

M.p.s were determined on a Kofler apparatus and are uncorrected. UV spectra were measured in EtOH (95%) with a JASCO Uvidec-510 spectrophotometer and IR spectra with a Perkin-Elmer 177 instrument. Optical rotations were taken on a JASCO DIP-181 polarimeter, and $[\alpha]_D$ values are given in units of 10^{-1} deg cm^2 g^{-1}. Mass spectra were acquired on a Finnigan MATTSQ70 spectrometer. NMR Spectra were recorded on a Bruker AC 250L spectrometer operating at 250.1 MHz for ^1H and 62.9 MHz for ^{13}C. Chemical shifts are in ppm (δ) from $SiMe_4$ as internal standard, and J values are given in Hz. DEPT and COLOC spectra were performed using the DEPT and COLOC pulse sequences of the AC 250L software. TLC and PLC were performed with Merck HF_{254} silica gel. For the purification procedure, we report the R_f-values in hexane–EtOAc (1:1) and CH_2Cl_2–MeOH (15:1), respectively.

Isolation and Purification of Metabolites **1**, **4** *and* **5**.—A strain of *Clitocybe clavipes* (CBS 126.44) was maintained on MPGA (malt, peptone, glucose, agar; 20:4:20:15 g dm^{-3}) slants and grown in 30 Roux flasks with the same medium for one month; the EtOAc extracts (0.7 g) were chromatographed on a column of flash silica gel with hexane–EtOAc (2:1) as eluent to obtain: clavilactone B (150 mg; R_f 0.5 and 0.8), clavilactone C (15 mg; R_f 0.4 and 0.3) and clavilactone A (50 mg; R_f 0.3 and 0.3). Some fractions were further purified by PLC in hexane–EtOAc (1:1).

Clavilactone A **1**.—Yellow solid, m.p. 176 °C; $[\alpha]_D$ +81 (*c* 0.2, MeOH); λ/nm 202 and 303 (ε/dm^3 mol^{-1} cm^{-1} 22 400 and 5300) (Found: C: 66.4; H, 5.4. $C_{16}H_{16}O_5$ requires C, 66.66; H, 5.59%); *m*/*z* (CI) 289 (MH^+); *m*/*z* (EI) 288 (M^+, 100%), 270 (12), 260 (10), 242 (17), 200 (25) and 187 (50); ^{13}C and ^1H NMR spectroscopic data are reported in Tables 1 and 2. Selected NOE experiments ([$^2H_6$]acetone + D_2O): {6-Hα} enhanced 7-Hβ (4.5%); {7-Hβ} enhanced 6-Hα (6%), 9-Hα (2%), 11-H (1%), 13-Hα (1%) and 16-H_3 (0.5%); {9-Hα} enhanced 7-Hβ (3.5%), 9-Hβ (18%), 10-Hα (2%) and 11-H (2.5%); {10-Hα} enhanced 9-Hα (2.5%), 9-Hβ (2%), 10-Hβ (12%) and 11-H (5%); {10-Hβ} enhanced 10-Hα (12%), 13-Hα (5%) and 13-Hβ (−0.55%); {11-H} enhanced 7-Hβ (1%), 9-Hα (1.5%), 10-Hα (5%) and 16-H_3 (2%).

Clavilactone B **4**.—Yellow solid, m.p. 76–79 °C; $[\alpha]_D$ −55 (*c* 0.15, $CHCl_3$); λ/nm 203, 250 and 310 (ε/dm^3 mol^{-1} cm^{-1}, 10 900, 11 790 and 1540) (Found: C, 66.9; H, 4.7. $C_{16}H_{14}O_5$

requires C, 67.12; H, 4.93%); *m/z* (EI) 288 (M^+ + 2, 15%), 286 (M^+, 25), 271 (12), 240 (15), 211 (38) and 199 (100; δ_H([$^2H_6$]acetone) 7.04 and 7.01 (2 H, d, *J* 10.0, 2- and 3-H), 5.92 (1 H, br s, 6-H), 5.38 (1 H, m, 11-H), 4.40 (1 H, br s, 7-H), 3.62 and 2.93 (2 H, br d, *J* 12.5, 13-H_2) 2.59 and 1.34 (2 H, m, 9-H_2), 2.35 and 2.28 (2 H, m, 10-H_2) and 1.51 (3 H, m, 16-H_3).

Clavilactone C **5**.—Yellow solid, m.p. 178–182 °C; $[\alpha]_D$ +110 (*c* 0.1, MeOH); λ/nm 310 (ε/dm^3 mol^{-1} cm^{-1} 6000) (Found: C, 62.9; H, 5.2. $C_{16}H_{16}O_6$ requires C, 63.15; H, 5.30%); *m/z* (CI, isobutane) 305 (MH^+) and 287 (MH^+ − 18); ^{13}C and ^{1}H NMR spectroscopic data are reported in Tables 1 and 2.

Acetylation of Clavilactone A **1**.—Clavilactone A (50 mg) was acetylated using standard conditions (pyridine–Ac_2O) and the solution was kept at 0 °C for 6 h. Purification by PLC in hexane–EtOAc (2:1) gave the diacetate **2** as a glassy solid, m.p. 85–90 °C; $[\alpha]_D$ +87 (*c* 0.1, MeOH); λ/nm 205 and 275 (ε/dm^3 mol^{-1} cm^{-1} 24 700 and 10 900); ν_{max}(KBr)/cm^{-1} 1770 (C=O), 1460, 1370 and 1175; *m/z* (CI, isobutane) 373 (MH^+), 317, 281, 279 (base peak) and 257; *m/z* (EI) 372 (M^+, 25%), 330 (M^+ − 42, 100), 288 (M^+ − 84, 55) and 260 (16); ^{13}C and ^{1}H NMR spectroscopic data are reported in Tables 1 and 2.

Methylation of Clavilactone A **1**.—Methylation (MeI, K_2CO_3, acetone) of compound **1** (100 mg) gave after standard work-up the dimethyl ether **3** (60 mg) as white crystals (diethyl ether–hexane), m.p. 168–172 °C; $[\alpha]_D$ + 111 (*c* 0.1, MeOH); λ/nm 205 and 305 (ε/dm^3 mol^{-1} cm^{-1} 12 800 and 2400) (Found: C, 68.2; H, 6.3. $C_{18}H_{20}O_5$ requires C, 68.34; H, 6.37%); *m/z* (CI, isobutane) 317 (MH^+); ^{13}C and ^{1}H NMR spectroscopic data are reported in Tables 1 and 2.

Interconversion between Clavilactone A **1** *and Clavilactone B* **4**.—Clavilactone B **4** (30 mg) was reduced with $NaBH_4$ in MeOH to give clavilactone A **1**, which, in turn, when treated with DDQ in toluene gave back compound **4**.

Reductive Acetylation of Compound **4**.—Clavilactone B **4** (10 mg) was stirred with Zn (15 mg) and Ac_2O (1.5 cm^3) at room temp. for 24 h; standard work-up followed by PLC on silica-gel in hexane–EtOAc (2:1) gave a compound identical (TLC, ^{1}H NMR) with **2**.

Biological Tests.—Antibacterial activity was tested using paper disks (6 mm diam.), soaked with test compounds (200, 100 and 50 μg) dissolved in EtOH which were placed in a suitable culture medium and poured into Petri dishes with *Bacillus cereus* (ATCC 10702), *B. subtilis* (ATCC 6633), *Sarcina lutea* (DMS 348) and *Saccharomyces cerevisiae* (NCYC 729), as test micro-organism. The antifungal activity of compounds **1**, **4** and **5** was tested using a direct bioautographic TLC assay. 50 μg Samples spotted on a silica gel TLC plate produced detectable inhibition of the growth of *Cladosporium* spp.

Crystal Structure Analysis of **3**.—X-Ray diffraction data were collected at room temperature (295 K) using a crystal measuring 0.80 × 0.65 × 0.32 mm on a Philips PW1100 diffractometer. Two standard reflections were monitored every 100 reflections, showing insignificant decay. 3923 Independent reflections with $3.5 \leqslant \theta \leqslant 60°$ of four octants ($\pm h$, $\pm k$, $+l$) were measured using the ω–2θ scan mode. 3747 Unique reflections were considered observed [$I \geqslant 2\sigma(I)$] and used in all subsequent calculations; Lorentz and polarization corrections were applied but no absorption or secondary extinction correction was deemed necessary.

Crystal data. $C_{18}O_5H_{20}$, M_r = 316.353, monoclinic, $F(000)$ = 1120, a = 9.258(3), b = 16.180(3), c = 11.103(2) Å, β = 103.97(3)°, V = 1614.0 Å^3, μ(Cu-Kα) = 7.42 cm^{-1}, space group $P2_1$, Z = 4 (2 molecules in the asymmetric unit), D_c = 1.3019 g cm^{-3}, λ(Cu-Kα) = 1.541 78 Å, colourless transparent prismatic crystals.

The structure was solved by direct methods using SHELXS86[15] and refined using full-matrix least-squares with SHELX76[16] while geometrical calculations were performed using the program PARST.[17] Hydrogen atoms were located at calculated positions and refined in riding mode. The final refinement converged at $R = R_w = 0.056$ (unit-weights), with the highest and lowest residual peaks in the Fourier difference of 0.44 and −0.40 e Å^{-3}. Attempts at establishing the absolute configuration were performed: final refinement of the two alternative enantiomers suggested no preference for either enantiomer. Examination of Bijvoet pairs[18] also did not yield any meaningful indication. Final positional parameters, anisotropic temperature factors, bond lengths and angles have been deposited with the Cambridge Crystallographic Data Centre.†

Molecular Modelling.—Molecular modelling and conformational search calculations were carried out using the MacroModel V4.0 package and the MM2* force field[12] on a SiliconGraphics Personal Iris 4D/35 workstation.

Acknowledgements

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† For details of the deposition scheme, see 'Instructions for Authors', *J. Chem. Soc., Perkin Trans. 1*, 1994, Issue 1.

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