Pergamon

PII: S0031-9422(98)00173-3

Phytochemistry, Vol. 49, No. 2, pp. 589–592, 1998

Printed in Great Britain
0031-9422/98/$-see front matter

SESQUITERPENES FROM THE NEMATICIDAL FUNGUS *CLITOCYBULA OCULUS*

IN HONOUR OF PROFESSOR G. H. NEIL TOWERS 75TH BIRTHDAY

WILLIAM A. AYER,[a] RUDONG SHAN,[a] LATCHEZAR S. TRIFONOV[a] and LEONARD J. HUTCHISON[b]

[a]Department of Chemistry, University of Alberta, Edmonton, Alberta, T6G 2G2, Canada
[b]Agriculture Canada Research Station, Lethbridge, Alberta, T1J 4B1, Canada

(*Received* 4 *December* 1997; *in revised form* 24 *February* 1998)

Key Word Index—*Clitocybula oculus*; *Aphelenchoides*; sesquiterpenes; clitocybulol; nematicidal activity; Cotton effect; nuclear magnetic resonance.

Abstract—Three sesquiterpenes, clitocybulol A (**1**), B (**2**) and C (**3**), with a new carbon skeleton were isolated from the Basidiomycete *Clitocybula oculus*. Their structures were determined by spectroscopic methods. The nematicidal activity on a fungus-feeding nematode *Aphelenchoides* sp. was examined, but no activity was observed.

INTRODUCTION

Soil nematodes are an important component of the soil microfauna. In one square meter of soil, approximately 1–3 million nematodes representing a biomass of 1.5–4.5 g are normally found [1]. Many are important plant parasites, while others are free-living saprophytes feeding upon bacteria. Some soil nematodes, however, are fungivorous, and feed upon the cytoplasm found in the hyphae of a diverse group of fungi [2, 3] resulting in the poor growth or death of these fungal hosts [4, 5]. In response, many fungi have independently evolved a variety of chemical defences known collectively as antifeedants, which protect their hyphal system against grazing by fungus-feeding nematodes [5, 6]. It has recently been observed that nematodes are quickly killed when exposed to cultures of the wood decaying Basidiomycete *Clitocybula oculus* (Peck) Singer [7]. This paper describes the metabolites produced by cultures of *C. oculus in vitro*, for which we suggest the names clitocybulol A (**1**), B (**2**), and C (**3**), respectively.

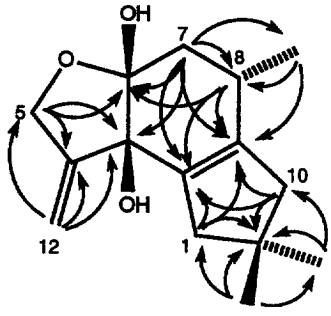
RESULTS AND DISCUSSION

The structure determinations are based on spectroscopic methods (including HREIMS, APT, HMQC, HMBC, COSY and ROESY experiments), although only pertinent results are provided herein.

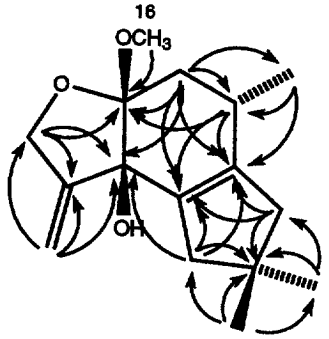
High resolution EI-MS measurements of clitocybulol A (**1**) indicated that its molecular composition is $C_{15}H_{22}O_3$. The ^{13}C NMR spectrum confirms the presence of 15 carbons. The structure of clitocybulol A (**1**) was elucidated by the HMBC correlations between 12-H_2 and C-3, C-4 as well as C-5, between 5-H_2 and C-4, C-12 as well as C-3/6, between 8-H and C-2, C-6, C-7, C-9, and C-13, and between 13-H_3 and C-7, C-8 and C-9 (see Fig. 1). The relative configuration of C-8 was established by the ROESY correlations between 13-H_3 and 5-H_α as well as 7-H_α, which is shown in Fig. 3. The ^1H-^1H coupling constants between 7-H_α and 7-H_β (14.2 Hz) and between 7-H_α and 8-H (2.2 Hz) suggest that both 7-H_α and 8-H are equatorial.

Clitocybulol B (**2**) may have been formed from clitocybulol A (**1**) when the extract of the culture fluid was left in methanol. The transformation of **1** to **2** when kept in methanol containing a catalytical amount of trifluoroacetic acid (TFA) at room temperature for 4 hours supports this assumption. The molecular composition of **2** was determined by high resolution EI-MS measurements to $C_{16}H_{24}O_3$. The pertinent HMBC correlations are shown in Fig. 1. The relative stereochemistry was established by the ^1H-^1H coupling constants between 7-H_α and 7-H_β (14.0 Hz) and between 7-H_α and 8-H (7.0 Hz) as well as the ROESY correlations which are shown in Fig. 2.

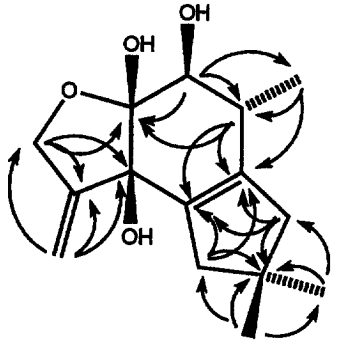
The high resolution EI-MS measurements of clitocybulol C (**3**) indicated that the molecular composition of **3** is $C_{15}H_{22}O_4$, and the NMR spectral data suggested that it contains one more hydroxyl group (at C-7) relative to clitocybulol A (**1**). HMBC correlations were observed between 12-H_2 and C-3, C-4, as well as C-5, between 5-H_2 and C-4, C-12, and C-3/6, between



1

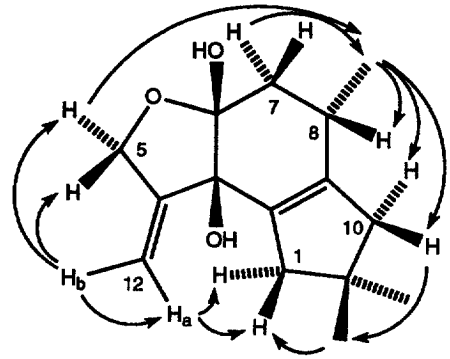


2

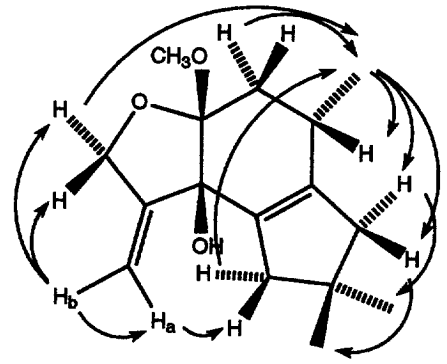


3

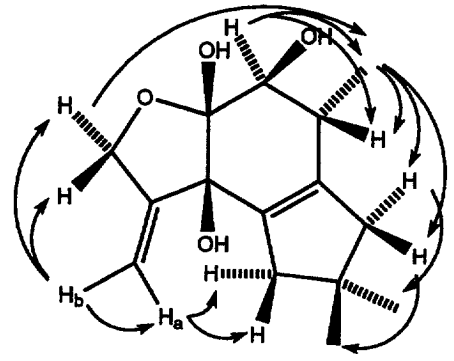
Fig. 1. Pertinent HMBC correlations of compounds **1**, **2** and **3**.



1



2

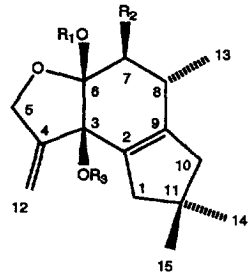


3

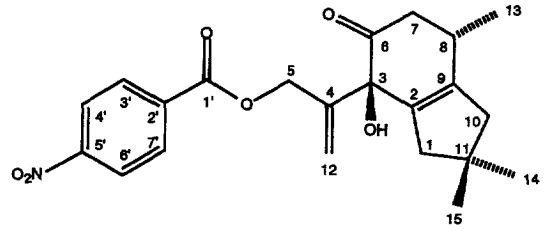
Fig. 2. Pertinent ROESY correlations of compounds **1**, **2** and **3**.

7-H and C-6, C-8 and C-13, and between 8-H and C-2, C-6, C-7, C-9, and C-13. The ^{1}H-^{1}H coupling constant between 7-H and 8-H (6.7 Hz) suggests that both 7-H and 8-H are equatorial, and this is supported by the ROESY correlations between 13-H_3 and 5-H_α, 7-H as well as 8-H, and between 7-H and 8-H as well as 13-H_3 as shown in Fig. 2.

All three compounds are sesquiterpenes with a carbon skeleton which has not previously been reported. The absolute configuration of the compounds **1**, **2** and **3**, each of which contains two double bonds, was based on the circular dichroic spectra of the compounds. Scott *et al* [8]. extended the octant rule to chiral olefins, by applying symmetry considerations. The chirality about the olefin chromophore through an olefin octant rule can be reflected by the Cotton effect sign of the principal $\pi \rightarrow \pi^*$ transition. The negative Cotton effects at 218 nm for **1**, 215 nm for **2** and 220 nm for **3** suggest that all the three compounds have the 3-*S* and 6-*R* configurations. Attempts to prepare the *p*-nitrobenzoyl derivative of clitocybulol B (**2**) which would be useful in confirming



1 R_1=R_2=R_3=H; clitocybulol A
2 R_1=CH_3, R_2=R_3=H; clitocybulol B
3 R_1=R_3=H, R_2=OH; clitocybulol C



4

of the absolute configuration failed, since the tertiary alcohol at C-3 did not react with *p*-nitrobenzoyl chloride. Instead, the ring opened and the C-6 ketone (**4**) was obtained. These compounds did not show nematicidal activity on *Aphelenchoides* sp., a fungus-feeding nematode, even at 1,000 ppm.

EXPERIMENTAL

The mass spectra were recorded with an AEI MS-50 mass spectrometer, while NMR spectra were recorded with a BRUKER WM-360 or VARIAN UNITY 500 spectrometer. UV spectra were obtained with a HEWLETT PACKARD Diode Array UV-Vis spectrometer (ICP) with a cell path of 1 mm, and IR spectra with a NICOLET 7199 Fourier Transform Infrared spectrometer (FTIR). The optical rotation was measured with a PERKIN ELMER 241 polarimeter with a cell path of 10 cm, and CD spectra were measured with a JASCO ORD-CD spectrometer with a cell path of 1 mm.

Clitocybula oculus, strain No. 378, was isolated and collected from fruiting bodies which were found on rotting wood in southern Ontario, Canada, and deposited at the culture collection of the University of Guelph, Ontario, Canada. For the production of the active metabolites, the fungus was maintained and cultivated on 10 liters of medium composed of (g/l): malt extract 20, yeast extract 2, and glucose 10. The culture was harvested after 60 days. After separation of the mycelium, the broth was concentrated *in vacuo* and extracted with EtOAc. Evaporation of EtOAc *in vacuo* gave an oily crude product (680 mg). Repeated flash chromatography on Silica gel 60 (hexane:EtOAc 1:1) and reversed-phase HPLC (μBondapak C_{18}, 35→100% MeOH in H_2O during 60 min.) yielded clitocybulol A (**1**) (23.0 mg), clitocybulol B (**2**) (7.1 mg), and clitocybulol C (**3**) (17.5 mg). The mycelium (30 g) was air-dried, ground, and extracted with acetone. Evaporation of acetone *in vacuo* gave an oily crude product (1.0 g). Flash chromatography on Silica gel 60 (hexane:EtOAc 4:1) and reversed-phase HPLC (μBondapak C_{18}, 90→100% CH_3CN in H_2O during 60 min.) gave ergosterol (30.8 mg) and fatty acids as the major metabolites, but no sesquiterpenes were detected.

Clitocybulol A (**1**) was obtained as white crystals. m.p. 89–91°C; $[\alpha]_D$ −80.0° ($CHCl_3$; *c* 0.45); UV (CH_3CN) λ_{max} (ϵ): 252 nm (1,322), 280 nm (297), 291 nm (276); CD: $\Delta\epsilon_{218}$ −8.62 (CH_3CN; *c* 0.01125); IR (chloroform cast): 3507, 3180, 3081, 2951, 2929, 2864, 2846, 1716, 1461, 1438, 1075, 951, 853 and 802 cm^{-1}; ^1H NMR (360 MHz, $CDCl_3$): δ 1.00 (3H, *s*, 14-H_3), 1.06 (3H, *d*, J=7.3 Hz, 13-H_3), 1.08 (3H, *s*, 15-H_3), 1.80 (1H, *dd*, J=14.2, 2.2 Hz, 7-H_α), 2.01 (1H, *m*, 10-H_β), 2.06 (1H, *m*, 7-H_β), 2.13 (1H, *m*, 1-H_β), 2.21 (1H, *m*, 10-H_α), 2.26 (1H, *m*, 1-H_α), 2.30 (1H, *m*, 8-H), 2.44 (1H, *s*, 6-OH), 3.08 (1H, *s*, 3-OH), 4.26 (1H, *dt*, J=13.1, 2.4 Hz, 5-H_α), 4.43 (1H, *dt*, J=12.9, 2.2 Hz, 5-H_β), 5.12 (1H, *t*, J=2.2 Hz, 12-H_b), 5.29 (1H, *t*, J=2.4 Hz, 12-H_a); ^{13}C NMR (125 MHz, $CDCl_3$): δ 149.4 (*s*, C-4), 143.2 (*s*, C-9), 130.0 (*s*, C-2), 107.6 (*t*,

C-12), 103.9 (*s*, C-6), 76.6 (*s*, C-3), 67.2 (*t*, C-5), 49.5 (*t*, C-10), 45.1 (*t*, C-1), 37.5 (*s*, C-11), 36.7 (*t*, C-7), 29.9 (*q*, C-14), 29.7 (*q*, C-15), 29.6 (*d*, C-8), 18.8 (*q*, C-13); HRMS, *m/z*: 250.15673 (12%, $C_{15}H_{22}O_3$ requires 250.15689), 232 (7%), 190 (77%), 175 (27%), 163 (100%), 135 (25%), 91 (16%), 69 (12%).

Clitocybulol B (**2**) was obtained as a pale purple oil.$[\alpha]_D$ −31.0° ($CHCl_3$; *c* 0.38); UV (CH_3CN) λ_{max} (ϵ): 216 nm (8,785), 253 nm (3,785), 280 nm (759), 291 nm (683); CD: $\Delta\epsilon_{215}$ −8.76 (CH_3CN; *c* 0.0095); IR (chloroform cast): 3555, 3425, 2951, 2925, 2865, 2835, 1716, 1462, 1438, 1075, 946, 932, 838 and 783 cm^{-1}. ^1H NMR (360 MHz, $CDCl_3$): δ 1.01 (3H, *s*, 14-H_3), 1.09 (3H, *d*, J=7.4 Hz, 13-H_3), 1.12 (3H, *s*, 15-H_3), 1.85 (1H, *dd*, J=14.0, 7.0 Hz, 7-H_α), 2.02 (1H, *m*, 10-H_β), 2.06 (1H, *m*, 7-H_β), 2.09 (1H, *m*, 1-H_β), 2.21 (1H, *m*, 10-H_α), 2.25 (1H, *m*, 1-H_α), 2.30 (1H, *m*, 8-H), 2.68 (1H, *s*, 3-OH), 3.32 (3H, *s*, 6-OCH_3), 4.30 (2H, *t*, J=2.4 Hz, 5-H_2), 5.09 (1H, *t*, J=2.5 Hz, 12-H_b), 5.24 (1H, *t*, J=2.5 Hz, 12-H_a); ^{13}C NMR (125 MHz, $CDCl_3$): δ 150.2 (*s*, C-4), 142.1 (*s*, C-9), 130.2 (*s*, C-2), 106.5 (*t*, C-12), 105.8 (*s*, C-6), 76.8 (*s*, C-3), 67.2 (*t*, C-5), 49.7 (*t*, C-10), 48.5 (*q*, C-16), 45.1 (*t*, C-1), 37.3 (*s*, C-11), 30.8 (*t*, C-7), 29.8 (*q*, C-14), 29.6 (*q*, C-15), 29.5 (*d*, C-8), 19.0 (*q*, C-13); HRMS, *m/z*: 264.17257 (13%, $C_{16}H_{24}O_3$ requires 264.17255), 217 (7%), 191 (34%), 190 (100%), 189 (16%), 175 (32%), 164 (13%), 163 (98%), 135 (16%), 91 (16%), 69 (17%).

Clitocybulol C (**3**) was obtained as a yellowish oil.$[\alpha]_D$ −57.7° ($CHCl_3$; *c* 0.36); UV (CH_3CN) λ_{max} (ϵ): 204 nm (12,400), 213 nm (12,520), 252 nm (3,183), 258 nm (3,124), 291 nm (1,045); CD: $\Delta\epsilon_{220}$ −13.31 (CH_3CN; *c* 0.010); IR (chloroform cast): 3416, 2950, 2925, 2865, 2837, 1463, 1436, 1381, 1269, 1231, 1100, 931, 878, 855 and 756 cm^{-1}; ^1H NMR, (360 MHz, $CDCl_3$): δ 1.01 (3H, *s*, 14-H_3), 1.07 (3H, *d*, J=6.9 Hz, 13-H_3), 1.09 (3H, *s*, 15-H_3), 2.04 (1H, *m*, 10-H_β), 2.08 (1H, *m*, 1-H_β), 2.24 (1H, *m*, 10-H_α), 2.29 (1H, *m*, 1-H_α), 2.52 (1H, *qd*, J=6.7, 1.5 Hz, 8-H), 4.02 (1H, *d*, J=6.7 Hz, 7-H), 4.29 (1H, *dt*, J=12.9, 2.5 Hz, 5-H_α), 4.45 (1H, *dt*, J=12.9, 2.5 Hz, 5-H_β), 5.12 (1H, *t*, J=2.2 Hz, 12-H_b), 5.30 (1H, *t*, J=2.5 Hz, 12-H_a); ^{13}C NMR (125 MHz, $CDCl_3$): δ 149.0 (*s*, C-4), 140.9 (*s*, C-9), 130.0 (*s*, C-2), 107.1 (*t*, C-12), 103.0 (*s*, C-6), 77.9 (*s*, C-3), 71.9 (*d*, C-7), 67.3 (*t*, C-5), 49.8 (*t*, C-10), 44.8 (*t*, C-1), 37.7 (*s*, C-11), 35.5 (*d*, C-8), 29.6 (*q*, C-14), 29.5 (*q*, C-15), 10.9 (*q*, C-13); HRMS, *m/z*: 266.15193 (16%, $C_{15}H_{22}O_4$ requires 266.15179), 248 (14%), 204 (14%), 203 (73%), 191 (80%), 164 (24%), 163 (100%), 135 (34%), 91 (18%), 69 (19%).

p-Nitrobenzoyl ketone (**4**): A solution of 3.3 mg (0.0125 mmol) of clitocybulol B (**2**) in 1 ml of methylene chloride was treated with 9.7 μl (0.07 mmol) of triethylamine, 1.0 mg of 4-dimethylaminopyridine, and 6.3 mg (0.0340 mmol) of *p*-nitrobenzoyl chloride. The mixture was stirred at 35°C for 4 hours, then was poured into 40 ml of ether and washed with 10% Na_2CO_3, 10% HCl, and brine. The organic layer was dried over $MgSO_4$ and the ether was removed *in vacuo*. Purification by HPLC (μBondapak C_{18}, 60→100% MeOH in H_2O during 60 min.) gave 1.0 mg of **4** as a colorless oil.$[\alpha]_D$ −56.0° ($CHCl_3$; *c* 0.10); UV (CH_3CN) λ_{max} (ϵ): 259 nm (6,872); IR (chloroform cast): 3463, 2954, 2925, 2864, 2850, 1725, 1529, 1410, 1378, 1320, 1271, 1143, 1115, 976 and 784 cm^{-1}; ^1H NMR, (360 MHz, $CDCl_3$): δ 1.07 (3H, *s*, 14-H_3), 1.10 (3H, *d*, J=6.8 Hz, 13-H_3), 1.13 (3H, *s*, 15-H_3), 2.08 (1H, *m*, 7-H), 2.30 (2H, *m*, 1-H and 10-H), 2.44 (2H, *m*, 1-H and 10-H), 2.72 (2H, *m*, 7-H and 8-H), 4.86 (1H, br. *d*, J=13.0 Hz, 5-H), 4.95 (1H, br. *d*, J=13.0 Hz, 5-H), 5.46 (1H, br. *s*, 12-H), 5.52 (1H, br. *s*, 12-H), 8.11 (2H, *dt*, J=9.0, 2.1 Hz, 3′-H and 7′-H), 8.27 (2H, *dt*, J=9.0, 1.9 Hz, 4′-H and 6′-H); HRMS, *m/z*: 399.16725 (13%, $C_{22}H_{25}NO_6$ requires 399.16818), 232 (66%), 217 (24%), 204 (23%), 190 (100%), 175 (62%), 163 (17%), 150 (85%), 104 (48%), 91 (36%), 69 (21%).

Acknowledgements—We thank the Natural Sciences and Engineering Research Council of Canada for financial support. We also thank Dr. T.T. Nakashima and Dr. G.E. Bigam for assistance with the NMR experiments, and Prof. G.L. Barron for supplying the culture of *C. oculus*.

REFERENCES

1. Wallwork, J. A., *Ecology of Soil Animals*. McGraw-Hill Publishing Co. Ltd., London, 1970, p. 283.
2. Mankau, R. and Mankau, S. K., in *Soil Organisms*, eds. J. Doeksen and J. Van der Drift. North-Holland Publishing Co., Amsterdam, 1963, pp. 271–280.
3. Townshend, J. L., *Can. J. Microbiol.*, 1964, **10**, 727–737.
4. Riffle, J. W., *Phytopathology*, 1967, **57**, 541–544.
5. Riffle, J. W., in *Mycorrhizae: Proc. 1st North American Conference on Mycorrhizae*, ed. E. Hacskaylo. USDA Forest Service Misc. Publ. 1189, Washington, D.C., 1971, pp. 97–113.
6. Hutchison, L. J., Madzia, S. E. and Barron, G. L., *Can. J. Bot.*, 1996, **74**, 431–434.
7. Hutchison, L. J. and Barron, G. L., Unpublished data.
8. Scott, A. I. and Wrixon, A. D., *Tetrahedron*, 1970, **26**, 3695–3715.