Pergamon

PII: S0031-9422(98)00258-1

Phytochemistry Vol. 49, No. 8, pp. 2591–2593, 1998

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

TINTINNADIOL, A SPHAEROANE DITERPENE FROM FRUITING BODIES OF *MYCENA TINTINNABULUM*

MICHAELA ENGLER,† TIMM ANKE† and OLOV STERNER‡*

†LB Biotechnologie der Universität, Paul-Ehrlich-Str. 23, D-67663 Kaiserslautern, F.R.G. and
‡Division of Organic Chemistry 2, Lund University, P.O.Box 124, S-221 00 Lund, Sweden.

(*Received* 9 *December* 1997)

Key Word Index—*Mycena tintinnabulum*; *Tricholomataceae*; fruiting bodies; diterpene; sphaeroane; isolation; structure determination; tintinnadiol; biological activities.

Abstract—Antifungal metabolites were isolated from fruiting bodies of *Mycena tintinnabulum* and from mycelia grown in either complex media or on oak wood. Strobilurins were produced in fruiting bodies and in mycelial cultures, whereas a new diterpene, named tintinnadiol, was isolated only from the fruiting bodies. The structure of the new compound was determined by spectroscopic methods. It exhibited cytotoxic effects. © 1998 Elsevier Science Ltd. All rights reserved

INTRODUCTION

Basidiomycetes provide an interesting source of novel secondary metabolites with a variety of biological activities [1]. Mycelial cultures of several *Mycena* species have been described to produce the antifungal strobilurins and oudemansins [2]. However, little is known about the production of antibiotics during different stages of differentiation, namely in the fruiting bodies.

In the present study, vegetative mycelia and fruiting bodies of *Mycena tintinnabulum* were extracted separately, and the biologically active compounds isolated. The isolation, the structure elucidation, and the evaluation of the biological activities of a new diterpene are reported in this paper.

RESULTS AND DISCUSSION

Caps, stems and substrate (oak wood) containing vegetative mycelia of *Mycena tintinnabulum* ME96001 were collected in the natural habitat and extracted separately. Tintinnadiol, a new diterpene with a sphaeroane skeleton, and strobilurin D were isolated from the caps of the fruiting bodies and the substrate, but not from the stems. Purification of the extracts derived from the caps yielded 2.1 mg of tintinnadiol and 0.2 mg of strobilurin D, while 1.1 mg tintinnadiol and 1 mg strobilurin D were purified from the substrate. In contrast, tintinnadiol was not produced in fermentations in YMG-medium, nor in axenic cultures on sterilized oak wood derived from the same log on which the fruiting bodies grew.

In the EI mass spectrum of tintinnadiol the peak of the molecular ion was also the base peak, and high resolution measurements established that the elemental composition of the compound was $C_{21}H_{32}O_3$. The signals for all carbons and protons were visible in the 1D NMR spectra, and the lack of correlation for two protons in the HMQC spectrum suggested the presence of two exchangeable protons. The unsaturation index of tintinnadiol is 6, the NMR data clearly indicated that it contained a substituted benzene ring but no other sp^2 carbons, and it should consequently contain two additional rings. The structure was established by the analysis of the COSY and HMBC (see Fig. 1) correlations. The methoxy protons correlated to C-13, while 15-H_3 correlated to C-11, C-12 and C-13, showing that the two were adjacent substituents on the benzene ring. The attachment of C-10 to the aromat was indicated by the correlations between 10-H and C-8, C-9 and C-10, and C-10 was shown to be attached to C-1 (by COSY as well as HMBC correlations) and to C-4 because of the HMBC correlations from 17-H_3 (appearing as a singlet in the ^1H NMR spectrum) to C-3, C-4, C-5 and C-10. The 2-hydroxypropyl group was positioned on C-3 because of the HMBC correlations from 19-H_3 and 20-H_3 to C-3 as well as C-18, and the positioning of C-2 between C-1 and C-3 closing the 5-membered

*Author to whom correspondence should be addressed.

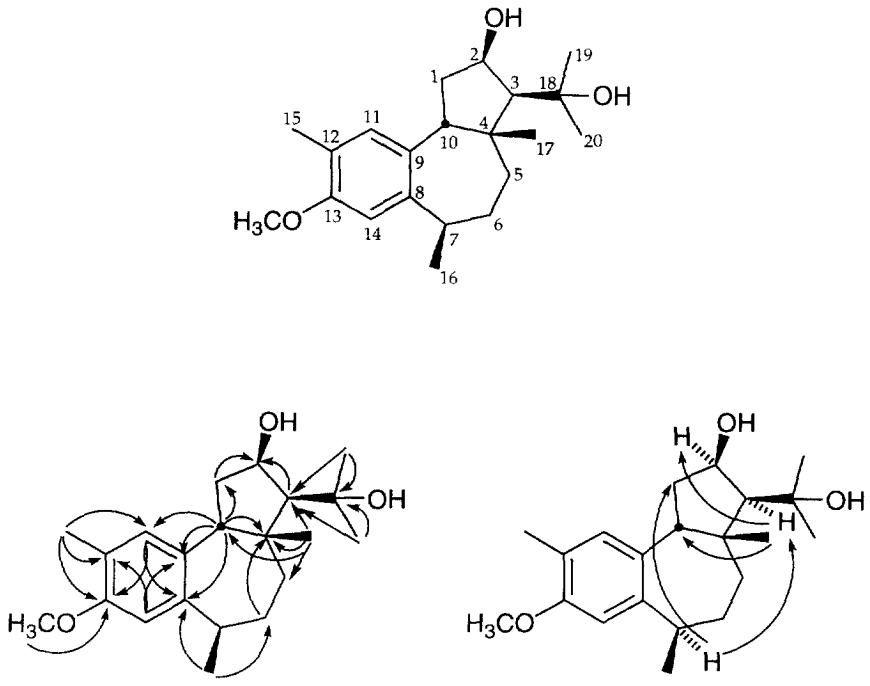

Fig. 1. Structure of tintinnadiol and numbering system for the sphaeroane skeleton (top), and pertinent HMBC (bottom left) as well as NOESY (bottom right) correlations observed with tintinnadiol in $CDCl_3$.

ring was supported by both COSY and HMBC correlations. The long-range ^1H-^{13}C correlations from 16-H_3 to C-8 as well as C-6 and the COSY correlations in the 5-H_2/6-H_2/7-H spin system, together with the HMBC correlations from both 5-H_2 and 6-H_2 to C-4, close the 7-membered ring and established the structure of tintinnadiol. The relative stereostructure was determined by the correlations observed in the NOESY spectrum (pertinent correlations are summarised in Fig. 1). The strong NOESY correlation between 17-H_3 and 10-H indicated that both were on the same side of the molecule, while the correlations observed between 7-H and 1-Hα as well as 3-H showed that 16-H_3 is also on this side. The correlation between 2-H and 3-H as well as the lack of correlation between 17-H_3 and 2-H or 3-H showed that 2-H and 3-H were situated on the other side compared to 17-H_3, and this situation permited a stabilising hydrogen bonding between the two hydroxyl groups (the presence of which was indicated by the sharpness of the signals for the two exchangeable protons in the ^1H NMR spectrum).

Tintinnadiol exhibited cytotoxic activities towards HL60 cells ($IC_{50}=10\ \mu g/ml$) and L1210 cells ($IC_{50}=40\ \mu g/ml$). In the agar diffusion assay, the compound showed neither antifungal nor antibacterial activity at concentrations up to 50 μg/disc.

To the best of our knowledge, this is the first time that a sphaeroane diterpene has been isolated from a fungus. This exclusive group of diterpenes has so far only been reported from an alga, *Sphaerococcus coronopifolius* [3, 4], to which there is no obvious evolutionary link from *Mycena tintinnabulum*.

EXPERIMENTAL

Producing organism

Fruiting bodies of *Mycena tintinnabulum* ME96001 were collected in Kaiserslautern (Germany) together with the substrate on which they were grown. Mycelial cultures were isolated from a spore print. The fungus was cultivated and maintained on YMG agar (yeast extract 0.4%, malt extract 1.0%, glucose 0.4%, and 1.5% agar, pH 5.5). The strain is deposited in the culture collection of the LB Biotechnology, University of Kaiserslautern.

Fermentation and isolation

Fermentations were carried out in 5 liter-Erlenmeyer flasks containing 2 l. of YMG medium at 22°C on a rotary shaker (120 rpm) for 6 weeks. Pieces of well grown agar plates of the same medium were used as inoculum. In addition, *Mycena tintinnabulum* was cultivated on its natural substrate. Sterilized oak wood cubes ($2\times2\times2$ cm) (280 g) were inoculated with pieces of a well grown mycelia culture and incubated in a Fernbach flask for 10 weeks at 22°C.

The caps (73 g) and the stems (19 g) of the collected fruiting bodies and the substrate with vegetative mycelia (55 g) were extracted separately, each with 1 l. of Me_2CO–MeOH (1:1). The crude extracts (cap: 532 mg; stems: 186 mg; substrate: 168 mg) were applied onto silica gel columns (Merck 60, 0.063–0.2 mm, 15 × 2.5 cm). The enriched extracts were obtained upon elution with cyclohexane-EtOAc (8:2). The final purification was achieved by preparative HPLC on LiChrosorb Diol (7 μm, column 250 × 25 mm, flow rate 5 ml min^{-1}) and elution with cyclohexane-tert-butyl methyl ether (7:3). The purification procedure of the metabolites from the mycelia (90 g) grown in YMG medium and on oak wood cubes (285 g) was carried out as described above.

Tests for biological activities

In the agar diffusion assay [5], the antifungal and antibacterial activities of tintinnadiol were tested against the following microorganisms: *Penicillium notatum*, *Mucor miehei*, *Nematospora coryli*, *Paecilomyces variotii*, *Bacillus brevis*, *Bacillus subtilis*, *Micrococcus luteus* and *Enterobacter dissolvens*. The cytotoxic activity of tintinnadiol was tested with HL60 and L1210 cells as described previously [6].

Spectroscopy

^1H NMR (500 MHz) and ^{13}C NMR (125 MHz) were recorded at room temp. with a Bruker ARX500 spectrometer with an inverse multinuclear 5 mm probehead equipped with shielded gradient coil. The spectra were recorded in $CDCl_3$ and the $CHCl_3/CDCl_3$ signals (7.26 and 77.0 ppm, respectively) were used as reference. The chemical shifts (δ) are given in ppm, and the coupling constants (J) in Hz. COSY, HMQC and HMBC experiments were recorded with gradient enhancements using sine shaped gradient pulses. For the 2D heteronuclear correlation spectroscopy the refocusing delays were optimised for $^1J_{CH} = 145$ Hz and $^nJ_{CH} = 10$ Hz. The raw data were transformed and the spectra were evaluated with the standard Bruker UXNMR software (rev. 941001). Mass spectra were recorded with a Jeol SX102 spectrometer, while the UV and the IR spectra were recorded with a Perkin Elmer λ 16 and a Bruker IFS 48 spectrometer. The melting point (uncorrected) were determined with a Reichert microscope, and the optical rotations were measured with a Perkin-Elmer 141 polarimeter at 22°C.

Tintinnadiol was obtained as colourless crystals from EtOH, mp 178–181°. $[\alpha]_D^{22} - 40°$ ($CHCl_3$; *c* {0.2}). UV λ_{max}^{MeOH} nm (log ϵ): 229 (3.84), 280 (3.27), 285 (3.26). IR ν_{max}^{KBr} cm^{-1}: 3400, 2930, 1615, 1575, 1505, 1460, 1410, 1210, 1050, 885. EIMS (probe) 70 eV, *m/z* (rel. int.): 332 [M]+ (100), 314 (8), 299 (9), 256 (19), 241 (22), 230 (94), 215 (66), 187 (28), 163 (26). High-resolution on the peak 332 [M] + : 332.2369; $C_{21}H_{32}O_3$ requires 332.2351. ^1H NMR (500.135 MHz, $CDCl_3$); δ 6.85 (1H, *s*, H-11), 6.73 (1H, *s*, H-14), 4.77 (1H, *m*, H-2), 3.83 (3H, *s*, CH_3O-13), 3.70 (1H, *s*, HO-2), 3.33 (1H, *t*, $J_{1-9} = 10$, H-10), 3.01 (1H, *m*, H-7), 2.63 (1H, *s*, HO-18), 2.17 (3H, *s*, H-15), 2.02 (2H, *m*, H-1), 1.92 (1H, *d*, $J_{2-3} = 3.3$ Hz, H-3), 1.77 (1H, *m*, H-6β), 1.58 (1H, *m*, H-5α), 1.53 (3H, *s*, H-19), 1.43 (3H, *s*, H-20), 1.40 (3H, *s*, H-17), 1.32 (3H, *d*, $J_{7-16} = 6.6$ Hz, H-17), 1.17 (1H, *m*, H-6α), 1.13 (1H, *m*, H-5β). ^{13}C NMR (125.759 MHz, $CDCl_3$): 156.8 (C-13), 139.9 (C-8), 113.4 (C-11), 133.0 (C-9), 123.3 (C-12), 107.8 (C-14), 76.0 (C-2), 75.0 (C-18), 57.8 (C-10), 55.4 (CH_3O-13), 52.9 (C-3), 42.7 (C-4), 40.1 (C-1), 36.7 (C-5), 33.2 (C-19), 32.6 (C-6), 32.1 (C-17), 31.2 (C-7), 29.2 (C-20), 19.4 (C-16), 15.5 (C-15).

Acknowledgements—Financial support from the Deutsche Forschungsgemeinschaft and the Swedish Natural Science Research Council is gratefully acknowledged.

REFERENCES

1. Erkel, G. and Anke, T. Products of secondary metabolism in *Biotechnology*, eds. H. Kleinkauf and H. von Döhren, Vol. 7. VCH-Verlag, Weinheim, 1997, pp. 490–533.
2. Anke, T., *Can. J. Bot.*, 1995, **73,** 940.
3. Cafieri, F., de Napoli, L., Fattorusso, E. and Santacroce, C., *Phytochemistry*, 1987, **26,** 471.
4. Cafieri, F., Fattorusso, E., di Blasio, B. and Pedone, C., *Tetrahedron Lett.*, 1981, **22,** 4123.
5. Zähner, H., *Biologie der Antibiotika*. Springer-Verlag, Berlin, Heidelberg, 1965.
6. Zapf, S., Hossfeld, M., Anke, H., Velten, R. and Steglich, W., *J. Antibiot.*, 1995, **48,** 36.