Coprinol, a New Antibiotic Cuparane from a *Coprinus* Species

Martin Johansson[a], Olov Sterner[a,*], Harald Labischinski[b] and Timm Anke[c,*]

[a] Division of Organic Chemistry 2, Chemical Center, University of Lund, P. O.Box 124, S-221 00 Lund, Sweden. Fax: +46 46 222 8209. E-mail: Olov.Sterner@orgk2.lth.se
[b] Bayer AG, PH-Research Antibacterial Therapy I, D-42096 Wuppertal
[c] Institut für Biotechnologie und Wirkstoff-Forschung IBWF e. V., Erwin-Schrödinger-Str. 56, D-67663 Kaiserslautern, Germany. Fax: +49 631 205 2999. E-mail: anke@rhrk.uni-kl.de

* Authors for correspondence and reprint requests

Z. Naturforsch. **56c,** 31–34 (2001); received October 23/November 6, 2000

Basidiomycete, *Coprinus* sp., Antibacterial Antibiotic

Coprinol, a new antibacterial cuparane, was isolated from fermentations of a *Coprinus* sp. Its biological activities were investigated and its structure was elucidated by spectroscopic methods. The new antibiotic exhibited activitiy against multidrug-resistant Gram-positive bacteria *in vitro*. Two derivatives were synthesized and their activities compared to the parent compound.

Introduction

Bacterial resistance to commonly used antibiotics is considered a severe threat to public health. Therefore, new chemical entities which can overcome the widespread resistance mechanisms are currently very much sought after. In our ongoing screening for new metabolites from higher fungi we detected that one of our *Coprinus* strains produced an antibacterial antibiotic with interesting activities against Gram-positive multiresistant strains including penicillin resistant pneumococci (PRSP), methicillin and quinolone resistant staphylococci (MRSA, QRSA), vancomycin resistant enterococci (VREF) and vancomycin intermediate resistant staphylococci (VISA). The compound had no activity against Gram-negative species.

Experimental

General

^{1}H NMR (500 MHz) and ^{13}C NMR (125 MHz) were recorded at room temperature with a Bruker ARX500 spectrometer with an inverse multinuclear 5 mm probehead equipped with a shielded gradient coil. The spectra were recorded in $CDCl_3$, or $CDCl_3$ containing 5% CD_3OD, and the solvent signals for $CHCl_3$ and $CDCl_3$ (7.26 in the ^{1}H NMR spectrum and 77.0 ppm in the ^{13}C NMR spectrum) were used as reference. 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 rotation measured with a Perkin-Elmer 141 polarimeter at 22 °C.

Coprinus sp. strain 90160

Mycelial cultures of *Coprinus* sp. strain 90160 were derived from tissue plugs of young fruit bodies. The wood-inhabiting species showed all characteristics of the genus, the species however, could not be identified. The culture and voucher specimen of the fruiting bodies are deposited in the culture collection of LB Biotechnology, University of Kaiserslautern. For maintenance on agar slants the fungus was grown on YMG medium (g/l): Yeast extract 4, malt extract 10, glucose 4, pH 7.0.

Fermentation and isolation of coprinol (**1a**)

Fermentations were carried out in 20 l of MGPY medium composed of (g/l): Yeast extract

0939–5075/2001/0100–0031 $ 06.00

1, maltose 20, glucose 10, peptone 2, KH_2PO_4 0.5, $MgSO_4 \times H_2O$ 1, $FeCl_3$ 0.01, $ZnSO_4 \times H_2O$ 0.0018, $CaCl_2 \times H_2O$ 0.074. in a Biolafitte C6 fermenter at 22 °C with an aeration rate of 2 l/min and agitation (120 rpm). A well grown culture of *Coprinus* sp. 90160 in the same medium (250 ml) was used as inoculum. During fermentation 100 ml samples were taken. The culture fluid was separated by filtration from the mycelia and the insoluble ingredients and then extracted with 100 ml of ethyl acetate. The residue obtained after evaporation of the organic solvent was taken up in 0.5 ml of methanol. 25 µl of the concentrated solutions were assayed for antibacterial activity in the agar plate-paper disc diffusion assay using *Bacillus brevis* as test organism. After 20 days of fermentation the culture broth (17 l) was separated from the mycelia and passed through a column (18 × 11 cm) containing Mitsubishi Diaion HP 21 adsorber resin. The column was washed with water and the compounds were eluted with 2,5 l of methanol. The methanol eluate was concentrated and the crude product (4 g) was applied onto a silica gel column (Merck 60, 0.063–0.2 mm; 12 × 5.5 cm). Elution with cyclohexane – ethyl acetate 3:7 yielded 602 mg of an enriched product containing **1a**. Final purification was achieved by preparative HPLC on Nucleosil C18 [7 µm; column 250 × 25 mm; flow rate 5 ml/min]. Elution with water-methanol 35:65 v/v yielded 92 mg of coprinol (**1a**).

Coprinol

Coprinol (**1a**) was obtained as an yellowish oil, $[\alpha]^D$ + 42° (*c* 1.0 in $CHCl_3$: CH_3OH 19:1). UV (MeOH), λmax (ε): 288 nm (3,500). IR (KBr): 3425, 2925, 1735, 1570, 1455, 1415, 1260, 1190, 1160, 1075, 1010 and 915 cm^{-1}. ^1H NMR (500 MHz, $CDCl_3$: CD_3OD 19:1): 6.442 and 6.436, s, 2-H and 5-H; 3.42, s, 10-H; 2.06, s, 15-H_3; 2.02, d, $J_{8a\text{-}8b}$=11.9, 8-Ha; 1.76, d, $J_{8a\text{-}8b}$=11.9, 8-Hb; 1.14, s, 14-H_3; 0.84, s, 12-H_3; 0.66, s, 13-H_3. ^{13}C NMR (125 MHz, $CDCl_3$: CD_3OD 19:1): 147.7 C-4; 145.2 C-1; 129.0 C-6; 123.8 C-3; 117.3 C-2; 112.5 C-5; 103.2 C-9; 83.7 C-10; 50.4 C-11; 44.3 C-7; 41.1 C-8; 23.7 C-13; 18.0 C-12; 17.0 C-14; 15.5 C-15. EIMS (70 eV), *m/z* (rel. int.): 264.1355 (100%, M^+, $C_{15}H_{20}O_4$ requires 264.1361), 246 (12%), 231 (31%), 218 (28%), 205 (38%), 191 (79%), 177 (24%), 175 (35%), 164 (42%), 151 (39%).

Acetylation of coprinol

The acetates **1b** and **1c** were prepared by acetylating coprinol (**1a**) according to the following procedure: 7.5 mg (0.028 mmol) **1a** was dissolved in 250 µl of pyridine (dried over 4Å molecular sieves) and 8.9 µl (0.094) acetic anhydride was added. The solution was stirred at room temperature under nitrogen atmosphere over night. Ethanol was added and the reaction mixture was concentrated under reduced pressure. Flash chromatography (heptane/ethyl acetate 4:1, silica gel) afforded 5.2 mg (56%) of the diacetylated derivative **1b** and 4.4 mg (44%) of the triacetylated derivative **1c**.

Compound **1b** was obtained as a colourless oil, $[\alpha]^D$ + 16.2 (*c* 0.5 , $CHCl_3$). ^1H NMR (500 MHz, $CDCl_3$): 6.71, s; 6.89, s; 4.62, d, *J*=1.8 Hz; 3.56, s; 2.30, s; 2.24, d, *J*= 12.1 Hz; 2.16, s; 2.10, s; 1.93, dd, *J*=12.1 Hz, *J*=1.8 Hz; 1.56, s; 1.28, s; 0.99, s; 0.77, s. ^{13}C NMR (125 MHz, $CDCl_3$): 171.9, 169.5, 149.5, 142.7, 129.9, 128.8, 119.3, 118.0, 103.8, 85.9, 50.2, 44.5, 40.7, 24.1, 20.9, 20.8, 19.6, 17.2, 15.9. LREIMS (*m/z*) 348, 306, 264, 233, 191, 164, 85, 83.

Compound **1c** was obtained as a colourless oil, [α]D + 2.1 (*c* 0.4 , $CHCl_3$). ^1H NMR (500 MHz, $CDCl_3$): 6.71, s; 6.68, s; 5.15, d, *J*=1.4 Hz; 2.30, m; 2.29, s; 2.13, s; 2.09, s; 2.07, s; 1.28, s; 0.89, s; 0.80, s. ^{13}C NMR (125 MHz, $CDCl_3$): 169.5, 169.2, 167.8, 148.8, 142.9, 130.0, 129.0, 119.1, 117.9, 104.8, 83.5, 49.7, 39.9, 24.2, 21.5, 20.8, 20.5, 20.0, 17.3, 15.9. LREIMS (*m/z*) 390, 348, 306, 271, 246, 233, 191, 164, 83.

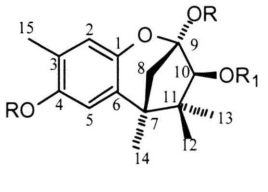
Biological tests

The assays for antimicrobial (Anke *et al.*, 1989) and cytotoxic activities (Zapf *et al.*, 1995) were carried out as described previously, if not mentioned otherwise in the text. For the determination of cytotoxic activities L1210 (lymphocytic leukemia, mouse ATCC CCL219) and Colo 320 (human colon adenocarcinoma DSMZ ACC 144) cells were used. L1210 and Colo 320 cells were grown in RPMI 1640 Medium (Gibco) containing 10% of fetal calf serum, 100 µg/ml streptomycin sulfate, and 65 µg/ml penicillin G/ml. The compounds to be tested were dissolved in methanol or ethanol and added to 200 µl of cell suspension ($4–5 \times 10^4$ cells/ml) in a cavity of a 96 well microtiter plate. The cells were incubated at 37 °C in a humidified

atmosphere containing 5% CO_2. Cell growth and lysis were observed in a microscope at 24 hour intervals for three days.

Results and Discussion

Coprinol (**1a**) (see Fig. 1 for chemical structures) was detected in a screening of basidiomycetes for the production of new antibacterial antibiotics. Its production and isolation is described in the experimental section. High resolution MS experiments suggested that its elemental composition is $C_{15}H_{20}O_4$, this was confirmed by the ^1H and ^{13}C NMR spectra and the structure of **1a** consequently contains 6 unsaturations. 4 of these could be assigned to a benzene ring, and as the NMR data did not suggest the presence of any other unsaturated bonds **1a** must contain two additional rings. The structure could be determined by 2D NMR, and especially the HMBC correlations summarised in Fig. 2 proved decisive. Although no correlation was observed between C-1



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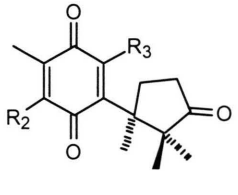

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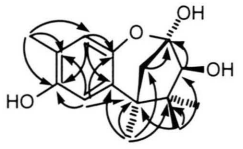
Fig. 1. a: R = R_1 = R_2 = R_3 = H;
b: R = Ac, R_1 = R_2 = H, R_3 = OH;
c: R = R_2 = Ac, R_2 = R_3 = OH.



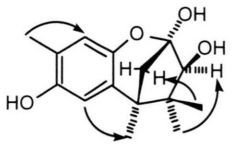


Fig. 2. Pertinent HMBC (left) and NOESY (right) correlations observed with coprinol (**1a**).

and C-9, the suggested ether is the only possibility that fits the MS and NMR data. The relative stereochemistry of coprinol (**1a**) was suggested by the NOESY correlations indicated in Fig. 2. No attempts were made to determine the absolute stereochemistry.

The lagopodins are structurally related cuparane sesquiterpenes isolated from *Coprinus lagopus* (Bollinger 1965) and *Coprinus cinereus* (Bu'Lock and Darbyshire 1976; Bastian 1985), and lagopodin A (**2a**), lagopodin B (**2b**) and lagopodin C (**2c**) have all been reported to possess antibacterial activity, However, they are different as they contain a benzoquinone moiety that can be suspected to be responsible for their biological activities. Although the molecular mechanism for the antibiotic activity of coprinol (**1a**) is less evident, the fact that the acetylated derivatives **1b** and **1c** are inactive suggests that the phenolic and/or hemiacetal hydroxyl group plays an important role.

Coprinol (**1a**) exhibits moderate antibiotic activities against Gram-positive bacteria with minimal inhibitory activities (MICs) of 20–50 μg/ml for *Bacillus brevis* ATCC 9999 and *Bacillus subtilis*

Table I. Minimal inhibitory concentrations (μg/ml) of coprinol **1a** and derivatives **1b** and **1c**.

Strain	MIC [μg/ml] Compound Coprinol **1a**	**1b**	**1c**
Escherichia coli 205127[1]	>100	>100	>100
Pseudomonas sp. 7966[1]	>100	>100	>100
Haemophilus influenzae Spain7[2]	>100	>100	>100
Streptococcus pneumoniae Sa8250[3]	6.25	>100	25
Streptococcus pneumoniae Sp670[3,4]	12.5	>100	>100
Enterococcus faecium L4001[5]	12.5	>100	>100
Staphylococcus aureus 48N[6,7]	25	>100	>100
Staphylococcus aureus MU50[6,7,8]	12.5	>100	>100
Staphylococcus aureus 25701[7]	25	>100	>100
Staphylococcus aureus LO3[6]	12.5	>100	>100

1 = Ciprofloxazin resistant; 2 = β-lactamase producer; 3 = erythromycin resistant (MLS8); 4 = penicillin resistant; 5 = vancomycin resistant (vanA); 6 = methicillin resistant; 7 = quinolone resistant; 8 = vancomycin intermediate resistant.

ATCC 6633. A panel of recent clinical isolates and reference strains was tested in a 96 well microtiterplate microdilution assay in Isosensitest broth (or Brain-heart infusion broth supplemented with 10% bovine serum in case of *S. pneumoniae* and 1% IsoVitaleX plus 10 mg/l hemin in case of *H. influenzae*) using an inoculum of 10^4–10^5 cells (Table I). No activity was found against Gram-negative strains and *Candida albicans*, but MIC values were obtained for Gram-positive multiresistant strains including penicillin resistant pneumococci (PRSP), methicillin and quinolone resistant staphylococci (MRSA, QRSA), vancomycin resistant enterococci (VREF) and vancomycin intermediate resistant staphylococci (VISA) in the range of 6.25–25 μg/ml. In the plate diffusion assay no antifungal activities were detected against *Penicillium notatum, Paecilomyces variotii, Mucor miehei,* and *Nematospora coryli* at 100 μg/disk. Moderate cytotoxic activities were observed starting from 25 μg/ml with L1210 cells (mouse) and none up to 100 μg/ml for Colo 320 cells (human). For compounds **1b** and **1c** with one exception (*S. pneumoniae* Sa8250, **1c**) no antibacterial activity could be detected up to 100 μg/ml in the microdilution assay. For lagopodin B MICs of 20–100 μg/ml and 10–20 μg/ml against the same strains of *B. brevis* and *B. subtilis* were observed. In the plate diffusion assay lagopodin B exhibited antifungal activities against *P. notatum, P. variotii, M. miehei,* and *N. coryli* at 100 μg/disk (Bastian 1985).

Acknowledgements

Financial support of the BMBF is gratefully acknowledged. We are indebted to Ms Petra Stark and Nadesha Neigel as well as to Mr. Frank Bauch for expert technical assistance.

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