Coprinol, a New Antibiotic Cuparane from a Coprinus Species

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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 multidrug-resistant bacteria in vitro. Two derivatives were synthesized and their activities compared to the parent compound.

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 CDC$_3$, or CDC$_3$ containing 5% CD$_3$OD, and the solvent signals for CHCl$_3$ and CDC$_3$ (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 $^J_{CH}$=145 Hz and $^3J_{CH}$=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 $\lambda$ 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 specimens 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 201 of MGPY medium composed of (g/l): Yeast extract 0939–5075/2001/0100–0031 $ 06.00 © 2001 Verlag der Zeitschrift für Naturforschung, Tübingen · www.znaturforsch.com · D
1, maltose 20, glucose 10, peptone 2, K₂HPO₄ 0.5, MgSO₄ × H₂O 1, FeCl₃ 0.01, ZnSO₄ × H₂O 0.0018, CaCl₂ × H₂O 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 (120 g) affording 5.2 mg of an enriched product containing 1a.

**Copolrirnol (1a)** was obtained as an yellowish oil, [α]D + 16.2 (c 0.5 , CHCl₃). 1H NMR (500 MHz, CDCl₃): 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. 13C NMR (125 MHz, CDCl₃): 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.

Compounds 1b and 1c were prepared by acetylation 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.

Compounds 1b was obtained as a colourless oil, [α]D + 2.1 (c 0.4 , CHCl₃). 1H NMR (500 MHz, CDCl₃): 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. 13C NMR (125 MHz, CDCl₃): 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 (4–5×10⁴ cells/ml) in a cavity of a 96 well microtitre plate. The cells were incubated at 37 °C in a humidified
atmosphere containing 5% CO₂. 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 C₁₅H₂₀O₄, this was confirmed by the ¹H and ¹³C 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 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.**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Coprinol 1a</th>
<th>MIC [μg/ml] Compound</th>
<th>1b</th>
<th>1c</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Escherichia coli</em> 205127°¹</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
<tr>
<td><em>Pseudomonas</em> sp. 7966¹</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
<tr>
<td><em>Haemophilus influenzae</em> Spain7²</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
<tr>
<td><em>Streptococcus pneumoniae</em> Sa8250³</td>
<td>6.25</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>25</td>
</tr>
<tr>
<td><em>Streptococcus pneumoniae</em> Sp670³⁴</td>
<td>12.5</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
<tr>
<td><em>Enterococcus faecium</em> L4001³</td>
<td>12.5</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em> 48N⁵,⁷</td>
<td>25</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em> MU50⁶,⁷,⁸</td>
<td>12.5</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em> 25701⁷</td>
<td>25</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em> LO3⁹</td>
<td>12.5</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
</tbody>
</table>

°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 microtiter plate 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

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