Coprinuslactone protects the edible mushroom *Coprinus comatus* against biofilm infections by blocking both quorum-sensing and MurA

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Originality-Significance Statement. The colonization of fruiting bodies was used as a guideline for the search of biofilm controlling compounds in clinical applications.

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Abstract

Pathogens embedded in biofilms are involved in many infections and are very difficult to treat with antibiotics because of higher resistance compared to planktonic cells. Therefore, new approaches for their control are urgently needed. One way to search for biofilm dispersing compounds is to look at defense strategies of organisms exposed to wet environments, which makes them prone to biofilm infections. It is reasonable to assume that mushrooms have developed mechanisms to control biofilms on their sporocarps (fruiting bodies). A preliminary screening for biofilms on sporocarps revealed that ectomycorrhizal fungi (living symbiotic with plant roots) harbored the highest bacterial diversity, but saprophytic ones (feeding on dead organic matter) showed few or no bacteria on their sporocarps. From the edible mushroom *Coprinus comatus* where no bacteria on the sporocarp could be detected (3R,4S)-2-methylene-3,4-dihdropentanoic acid 1,4-lactone, named coprinuslactone, was isolated. Coprinuslactone interfered with quorum-sensing in *Pseudomonas aeruginosa*, where it also reduced the formation of the pathogenicity factors pyocyanin and rhamnolipid B. Furthermore, it inhibited UDP-N-acetylglucosamine enolpyruvyl transferase (MurA), essential for bacterial cell wall synthesis. These two modes of action ensure the inhibition of a broad spectrum of pathogens on the fruiting body but may also be useful for future clinical applications.

Keywords: biofilm, mushroom, quorum-sensing, MurA, chemical ecology

Introduction

In nature most bacteria are settled in biofilms, a self-organizing and dynamic lifeform where the cells are embedded in polymers representing a protected means of growth that allows survival in hostile environments (Hall-Stoodley *et al.*, 2004). Biofilms are involved in up to 80% of all microbial infections in humans (Davies, 2003), from rhinosinusitis and urinary tract infections to infection on all type of implants (Burmolle *et al.*, 2010). Biofilms are typically connected with chronic conditions (Hentzer & Givskov, 2003) and can cause severe complications in infections (Herwaldt *et al.*, 2006). For the treatment of biofilm infections on implants antibiotic treatment at elevated doses is the first approach to control the infection, however, sometimes substitution of the implant is the only solution which is accompanied by complication risks of the surgery and high costs (Fux *et al.*, 2005). *Staphylococcus* spp. followed by *Pseudomonas aeruginosa* is usually related with many of these infections (Costerton *et al.*, 1999). When organized in biofilms bacterial cells resist clearance by the host immune system and display highly increased resistance to antimicrobial agents (Olsen, 2015). This protection, however, is reversible and lost when the biofilm is dissolved and planktonic cells are set free.

Due to the failure of available methods to treat biofilms infections, new therapies which specifically prevent biofilm formation or actively eradicate already established biofilms are urgently needed (Ranall *et al.* 2012, Estrela *et al.*, 2009). One promising target for these new drugs is quorum-sensing (QS), the communication of microbial cells to form and maintain biofilms (Schauder & Bassler 2001). Interfering with bacterial communication pathways leads to reduced biofilm formation and attenuates expression of virulence factors (Rasmussen *et al.*, 2006). Because quorum quenchers only stop

biofilm formation but do not kill the bacteria it is assumed that there is a lower pressure for the development of resistances, however, some resistance has been reported (Garcia-Contreras *et al.*, 2015). Some fungal metabolites capable of inhibiting QS and biofilm formation are already known, e.g. patulin and penicillic acid from *Penicillium* sp. active against *Pseudomonas aeruginosa* biofilms (Rasmussen *et al.*, 2005) or *cis*-cyclo (leucyl-tyrosyl) from a marine *Penicillium* sp. targeting *Staphylococcus epidermidis* biofilms (Scopel *et al.*, 2013).

One way to search for quorum-quenching or biofilm dissolving compounds is to look at organisms which are exposed to wet environments making them prone to biofilm infections. As it is reasonable to assume that these organisms have developed several strategies to control their biofilms we decided to look for fungi, used to have their sporocarps (fruiting body) exposed to humid air, as source for novel quorum-sensing inhibitors. A preliminary screening for biofilms on sporocarps revealed that ectomycorrhizal fungi (living symbiotic with plant roots) harbored the highest bacterial diversity, but saprophytic ones (feeding on dead organic matter) showed little or no association with bacteria. Screening of several of these fungi for antimicrobial and antibiofilm activities demonstrated the ability of saprophytic fungi to modulate biofilm colonization on their sporocarps (de Carvalho *et al.*, 2015). This approach led to the isolation of (3R,4S)-2-methylene-3,4-dihydroxypentanoic acid 1,4-lactone, named here coprinuslactone, from the fruiting bodies of the edible mushroom *Coprinus comatus*. This compound, known before only as a synthetic intermediate, was also found in vegetative mycelia and culture media extracts of isolates. Its biological activities and its mode of action are described here together with its proposed ecological role.

Results

Identification of the active compound

Extracts obtained after 42 days of *C. comatus* cultivation showed antimicrobial activity against all tested bacteria as listed in Materials and Methods. The active compound was purified, yielding approximately 2 mg L⁻¹ in BAF medium or 100 mg kg⁻¹ of mycelia or fruiting bodies. Mass spectra showed a peak at m/z 129 [M + H]⁺, ¹H NMR analysis revealed an exo-methylene moiety at δ_H 6.34 and 5.98, a doublet of a methyl group at δ_H 1.44 and two protons at δ_H 4.45 and 4.34. Using ¹H-¹³C-correlated NMR (Fig. 1A) spectra, structure elucidation led to its identification as 2-methylene-3,4-dihydroxypentanoic acid 1,4-lactone **1**. *Trans*-configuration of the compound was deduced from the resonances of the methyl group at δ_H 1.44 instead of 1.40 and of 4-H at δ_H 4.34 instead at 4.7-5.0 reported for the *cis*-isomer (Barbier & Benezra, 1983). Absolute configuration was determined from the negative sign of the optical rotation to be 3R,4S as reported for this compound prepared by asymmetric synthesis (Bernardi *et al.*, 1985). Compound **1** was therefore identified as (3R,4S)-2-methylene-3,4-dihydroxypentanoic acid 1,4-lactone and named coprinuslactone after the producing fungus.

Together with **1** a second metabolite was isolated with [M+H]⁺ of 131 and the composition C₆H₁₀O₃. Its NMR spectra proved this compound to be dihydro-coprinuslactone **2**. The relative configuration of the ring substituents were determined by comparison with NMR resonances of known compounds. NMR data of (2R,3R,4S)- and

(2S,3R,4S)-2-methyl-3,4-dihydroxypentanoic acid 1,4-lactone, 5S,3'*R*,4'S,5'S- and 5S,3'S,4'S,5'S-hydroxyancepsenolide as well as (2S*,3R*,4S*)-2-n-hexadecyl-3,4-dihydroxypentanoic acid 1,4-lactone (Lorenzo *et al.*, 2006) revealed that a *trans*-configuration of the 3-OH to the 4-methyl group causes a shielding of about δ_H 0.3-0.7 ppm and a deshielding of about δ_C 5 ppm compared to a *cis*-arrangement. 1H NMR and optical rotation data of both the (2R,3R,4S)-*trans,trans*- and (2S,3R,4S)-*cis,trans*-coprinuslactone reported by Bernardi *et al.* 1985 led to the identification of **2** as (2S,3R,4S)-2-methyl-3,4-dihydroxypentanoic acid 1,4-lactone (Fig. 1). The dihydro-derivative **2** of coprinuslactone, not yet known as natural product, did not show any bioactivity in our tests.

Coprinuslactone does not prevent biofilm formation but dissolves existing ones

Optical density (OD₆₀₀) measurements indicated a minimal inhibitory concentration (MIC) of coprinuslactone of 150 $\mu g\ mL^{-1}$ for *Pseudomonas aeruginosa* PA14. However, after 24 h incubation in fresh LB agar 294 CFU were counted indicating a bacteriostatic effect. At the concentrations of 300 $\mu g\ mL^{-1}$ and 600 $\mu g\ mL^{-1}$, coprinuslactone acted as an antibiotic and no CFUs formed on the plates. Coprinuslactone had also moderate MICs against several bacteria from 37.25 to 150 $\mu g\ mL^{-1}$ (Tab. S1) but no activity was observed against yeasts. To verify quorum quenching properties of coprinuslactone, serial dilutions started at 75 $\mu g\ mL^{-1}$ because within this concentration range, there was no effect on bacterial growth, as shown in Fig. 2.

After establishing MICs for *P. aeruginosa* PA14 and *S. aureus* DSM 1104 biofilm inhibition at subtoxic concentrations was evaluated. For both strains biofilm formation

was not inhibited when coprinuslactone was employed below its MIC. This was different for *in vitro* established biofilms where 100 $\mu\text{g mL}^{-1}$ of coprinuslactone were effective in damaging 90% $\pm$ 10% of the staphylococcal biofilms. When 50 $\mu\text{g mL}^{-1}$ were used, still 65% $\pm$ 15% of the cells were stained red, demonstrating that even at sub-MIC-concentrations, coprinuslactone could cause cellular disturbance (Fig. 3 A-D). A similar effect was seen for *P. aeruginosa* PA14 where treatments with the established MIC of coprinuslactone (150 $\mu\text{g mL}^{-1}$) were effective in damaging 99.9% $\pm$ 0.3% of the *in vitro* developed biofilms with a remaining biofilm volume of only 1% $\pm$ 0.5% compared to the control. When biofilms were treated with 50% of the MIC, still a severe damage was found with a remaining biofilm volume of 7.5 $\pm$ 1% and 1% $\pm$ 0.6% of living bacterial cells. Reducing the treatment to 25% of MIC still decreased the biofilm volume to 15% $\pm$ 2% but 88% $\pm$ 5% of the cells remained alive (Fig. 3 E-H).

Coprinuslactone has two modes of action

As the activity of coprinuslactone against established biofilms had been demonstrated, its influence on *quorum sensing* systems was tested. When the *Escherichia coli* mutant MT102 (pSB403) - a reporter strain for *lux* genes of *Vibrio fischeri*, responding to short chain HSLs - was treated with 62.5 $\mu\text{g mL}^{-1}$ of coprinuslactone antibacterial activity was observed. Nevertheless, 3.75 $\pm$ 0.25 $\mu\text{g mL}^{-1}$ of coprinuslactone showed a complete abolishment of the QS response of the autoinducer 3-oxo-C₆-HSL. The *Pseudomonas putida* mutant F117 (pKR-C12) was less sensitive to coprinuslactone where 30 $\mu\text{g mL}^{-1}$ decreases the QS response of 3-oxo-C₁₂-HSL by 80% $\pm$ 5%. Again, 62.5 $\mu\text{g mL}^{-1}$ showed antibacterial activity. Having shown that coprinuslactone interferes at subtoxic concentrations with quorum-sensing in Gram-negative bacteria its effects on the

production of pathogenicity factors of *P. aeruginosa* PA14 was assessed. 75 $\mu\text{g mL}^{-1}$ of coprinuslactone were able to reduce the production of pyocyanin by $80\% \pm 3\%$ and of rhamnolipid B by 100%. When 37.5 $\mu\text{g mL}^{-1}$ of coprinuslactone were applied, $48\% \pm 5\%$ of pyocyanin production was inhibited, as well as $97.4\% \pm 2\%$ of rhamnolipid B and even treatments using 18.75 $\mu\text{g mL}^{-1}$ of coprinuslactone allowed the production of only $44\% \pm 10\%$ of rhamnolipid B but pyocyanin production reached almost 80% compared to the untreated control.

After establishing the interference of coprinuslactone with the quorum-sensing mechanisms in Gram-negative cells we determined its effects on cell morphology. Cells of *P. aeruginosa* PA14, treated with coprinuslactone, showed a high amount of intracellular electron dense bodies (Fig. S1, arrows 1- 5; 1D, arrows 2 and 3), which in general appeared to be in contact with the cytoplasmic membrane. These electron dense bodies were far less frequent in the methanol treated controls (Fig. S1, arrows 2 and 3). The appearance of extracellular fibrillar matter, similar to that seen in the chromosome area (Fig. S1, arrow 1), could be observed, undoubtedly representing leaking DNA. Coprinuslactone inhibited also the growth of *S. aureus* which cannot be explained by interference with its quorum-sensing system. Treatment of *Staphylococcus aureus* DSM 1104 with coprinuslactone caused a reduction of the cell diameter by 31% compared to the methanol treated control (Fig. S2 arrow 1). Interestingly, only very few cells were observed in the division state (Fig. S2, arrow 2). A myelin-like organization of the cytoplasmic membrane was strictly correlated with the lactone influence (Fig. S2, arrows 2 and 3).

These ultrastructural features, especially the leakage of DNA from the cells, indicated an influence of coprinuslactone on the integrity of the cell membrane. To elucidate this further we were looking at the effects of coprinuslactone on the activity of UDP-N-acetylglucosamine enolpyruvyl transferase (MurA), catalyzing the first step in [peptidoglycan](#) biosynthesis (Brown *et al.*, 1995). Because the MurA gene is conserved across bacteria (Gautam *et al.*, 2011) MurA of *P. aeruginosa* PA14 was cloned, overexpressed, purified and incubated with several concentrations of coprinuslactone. From these experiments an IC₅₀ (inhibitory concentration showing 50% of inhibition) of 10 µg mL⁻¹ was determined for coprinuslactone which was well below the MIC for *P. aeruginosa* PA14.

Coprinuslactone has low cytotoxicity

Above 3 µg mL⁻¹ coprinuslactone lowered cell viability in the MTT assay but even at 30 µg mL⁻¹ it did not kill all cells. Immunofluorescence images of treated cells stained with anti-α-tubulin and with DAPI revealed an increase of multinucleated cells (Fig. 3 I, arrows 1 and 2). Nevertheless, the majority of the cells still presented a normal microtubular distribution and nuclear morphology (Fig. 3 K, arrow 3) corroborating the finding from the MTT assay of cytostatic but not cytotoxic effects.

Discussion

Our working hypothesis was that organisms exposed to a wet environment are used to be threatened by biofilm infections and have therefore developed strategies to control

biofilm formation on their bodies. This hypothesis was confirmed by a preliminary screen for biofilms on basidiocarps collected in Northern Germany which revealed that ectomycorrhizal fungi harbored the highest biofilm diversity, but saprophytic ones showed little or no association with bacteria (de Carvalho *et al.*, 2015). Because no bacteria could be detected on the basidiocarps of *Coprinus comatus* collected at different sites in Germany we expected an antimicrobial compound being produced by the fungus. *C. comatus*, an edible mushroom of cosmopolitan occurrence and cultivated in China and Taiwan (Ju *et al.*, 2011), is known as a medicinal fungus. A number of biological activities, including anticancer (Zaidman *et al.*, 2008), antioxidant (Li *et al.*, 2010; Tsai *et al.*, 2009), nematocidal (Luo *et al.*, 2004) and analgesic activities (Ren *et al.*, 2012) have been reported. *C. comatus* fruiting bodies also display antimicrobial activity (Stojković *et al.*, 2013; Vamanu, 2013) but the underlying chemical compounds have not yet been identified. Activity-directed fractionation led to the isolation of (3R,4S)-2-methylene-3,4-dihydroxypentanoic acid 1,4-lactone, named coprinuslactone. It was detected both in its fruiting bodies contributing to the activity of this medicinal fungus, in mycelium and in fermentation broths of different isolates. Alpha-methylene-gamma-butyrolactones are mainly known from plants (Kitson *et al.*, 2009). Among the few reports from fungi are 2-methyl-2-pentene-4-olide **3** (Fig. 1 C), a flavor compound of *C. comatus* (Dijkstra & Wiken, 1976), and (3S,4R)-3-carboxy-2-methylene-heptan-4-olide **4** from the plant pathogen *Lasiodiplodia theobromae* (He *et al.*, 2004).

Coprinuslactone has bacteriostatic properties against both Gram-positive and Gram-negative bacteria. Because Gram-negative and Gram-positive bacteria have rather distinct quorum-sensing mechanisms the activity of coprinuslactone could not only be

explained by quorum-quenching and another mode of action had to be suspected. Membrane damage, causing cytoplasmic leakage, was clearly observed in *P. aeruginosa* PA14 micrographs. For *S. aureus* DSM 1104, myelin-like structures could be seen in treated cells, also observed after lysozyme treatment (Wecke *et al.*, 1982). Lysozyme catalyses hydrolysis of the β -1-4 bond between *N*-acetylmuramic acid and *N*-acetylglucosamine, the building blocks of the bacterial cell wall peptidoglycan (Chipman & Sharon, 1969). This could imply interference of coprinuslactone with peptidoglycan synthesis.

To confirm this hypothesis we looked for related compounds and their molecular targets. Closely related to coprinuslactone are tulipalin A **5** and B **6** (Fig. 1 C), first isolated from *Tulipa gesneriana* (Bergman *et al.*, 1967; Tschesche *et al.*, 1969). Tulipalins have antimicrobial activities and are recognized as chemical self-defense compounds, acting against microbial infections and insect predations (Shigetomi *et al.* 2010). The prime target of tulipalin B is MurA, (Shigetomi *et al.*, 2013). Because of its essential role in bacterial cell wall synthesis this enzyme has been evaluated as a target for antibacterial drugs, however, fosfomycin is still the only MurA inhibitor in clinical use (Bachelier *et al.*, 2006; Mendgen *et al.*, 2010). Moreover, MurA appears to be upregulated in *E. coli* and *Streptococcus suis* biofilms compared to planktonic cells, suggesting a possible role in biofilms resistance against antibiotics (Schembri *et al.*, 2003; Wang *et al.* 2012). Congrouly, the MurA-blocker fosfomycin combined with antibiotics has been successfully used to treat biofilms (Anderson *et al.*, 2013). Within this work we demonstrated that coprinuslactone binds MurA, inactivating the enzyme. However, considering the ultrastuctural modifications of the bacterial cells the second proposed

mechanism of action is membrane destabilization. In this case, coprinuslactone would interact with negatively charged lipids, causing membrane permeabilization.

Not only being an inhibitor of MurA, coprinuslactone also interferes with acyl-homoserinyl lactone (AHL) quorum-sensing autoinducers. It was able both to damage and to disperse *in vitro* grown biofilms of *P. aeruginosa* PA14. Coprinuslactone inhibited both QS systems of the mutants *E. coli* MT102 (pSB403) and *P. putida* F117 (pKR-C12) revealing *quorum quenching* properties. It is tempting to speculate that the γ -butyrolactone moiety of coprinuslactone, closely related to homoserine lactone, interacts with the active sites of acyl-homoserine lactone receptor proteins. Although QS inhibition could be confirmed, no initial biofilm inhibition was observed and the impact of coprinuslactone on the formation of virulence factors was assessed. *P. aeruginosa* AHL-mediated QS systems control not only biofilm formation but also the expression of several virulence factors such as pyocyanin, rhamnolipid B, cyanide and superoxide dismutase (Jakobsen *et al.*, 2013). Rhamnolipid B affects polymorphonuclear leukocytes (PMNs), causing necrosis and enabling *P. aeruginosa* persistence in lungs (Jensen *et al.*, 2007). Pyocyanin alters calcium concentrations in the cytoplasmic matrix of the epithelial cells, inhibiting their ciliary activity and leading to lung damage. It was reported that pyocyanin also inhibits the expression of interleukin (IL)-2 and its receptor and stimulates IL-8 release in human epithelial cells (Fuse *et al.*, 2013).

Using HPLC-MS-MS quantification, we showed that coprinuslactone inhibits the QS products pyocyanin and rhamnolipid B in *P. aeruginosa* PA14. Working with a *P. aeruginosa* PAO1 mutant, unable to synthesize rhamnolipids, Davey *et al.*, 2003, demonstrated that those molecules regulate biofilm architecture, without affecting initial

biofilm formation. This corroborates our data, where 3D reconstructions of the treated biofilms showed a volume decrease of approximately 85% under the influence of 0.5 MIC of coprinuslactone. Usually, a spectrophotometric method (Essar *et al.*, 1990) is used to measure pyocyanin. Here, a more sensitive HPLC-MS-MS method for quantification in culture media was established, revealing that coprinuslactone inhibited pyocyanin production by 80% even at 0.5 MIC. This confirms a recent study where synthetic autoinducer-mimics were found to antagonize pyocyanin production, however, no structure activity relations could be established (Morkunas *et al.*, 2012). The finding that coprinuslactone attenuated virulence factors of *P. aeruginosa* may have not only implications for the chemical ecology of the compound but also for any potential application in the treatment of biofilm infections. In this context it may be interesting that coprinuslactone also damaged *S. aureus* DSM 1104 biofilm, although no inhibition on biofilm formation was detected. Even at concentrations below the MIC this activity was observed. We hypothesize that coprinuslactone may also have an impact on virulence factors of *S. aureus* because attenuation of the prominent virulence factor α -hemolysin by a related α -methylene- γ -butyrolactone was recently reported (Kunzmann *et al.*, 2014).

Results obtained with the impedance method assay, showed no cytotoxicity of coprinuslactone towards eukaryotic cells. Cell morphology visualized by fluorescence microscopy suggested that coprinuslactone acts as a weak cytostatic agent. Contrary to coprinuslactone, it was reported that tulipalin A, lacking both the hydroxyl- and the methyl-moiety of coprinuslactone, possesses an IC_{50} value of $0.8 \mu\text{g ml}^{-1}$ (Perry & Brennan, 1997). It also causes contact allergies among workers in the tulip fields

(Lepoittevin *et al.*, 2009). Guinea pigs have been used for sensitization against allergenic compounds and both enantiomers of tulipalin A **5** showed positive reactions. On the other hand, when tulipalin B **6** and synthetic β -hydroxy- γ -methyl- α -methylene- γ -butyrolactone **1** were evaluated, the positive reactions were enantiospecific (Papageorgiou *et al.*, 1988).

With the data obtained within this work in combination with those reported for tulipalins some structure-activity relations can be proposed. The hydroxy group decreases the toxicity of tulipalin B **6** and coprinuslactone **1** against fungi and mammalian cells but increases at the same time the toxicity against bacteria. The methylene group is essential for biological activities, since (2*S*,3*R*,4*S*)-2-methyl-3,4-dihydroxypentanoic acid 1,4-lactone **2** did not show any activity in the tests.

Judging from its biological activities the ecological role of coprinuslactone is the defence of the basidiocarps of *C. comatus* against bacteria. Its failure to block formation of biofilms and its ability to dissolve them, hints to the control of the microbial community rather than to its prevention. This is in line with similar observations on other saprophytic fungi (de Carvalho *et al.*, 2015). Such characteristics together with its low cytotoxicity, activity against both Gram-negative and Gram-positive bacteria, and simple structure qualify coprinuslactone as a lead compound for the development of biofilm-dissolving drugs. Especially in combination with antibiotics this may be a novel route in controlling biofilm infections (Estrela & Abraham, 2010). Further investigations of its impact on mammals are needed in order to evaluate possible applications concerning biofilm infections in humans. A better knowledge on fungal interactions with their bacterial neighbours is a valuable approach to apply chemical ecology in drug discovery

programs (Hall-Stoodley et al., 2012). An intensive search in saprophytic fungi for their biofilm modulating compounds will not only lead to a deeper understanding of the chemical ecology between fungi and their attached bacteria but also to novel secondary metabolites which may find their way into clinical applications.

Experimental procedures

Strains of microorganisms

For antimicrobial assays *Bacillus cereus* DSM 626, *Escherichia coli* DSM 498, *Micrococcus roseus* DSM 20447, *Pseudomonas aeruginosa* PA14, *Staphylococcus aureus* DSM 1104, *S. epidermidis* ATCC 35984, *Streptococcus mutans* UA159, *Candida albicans* DSM 11225, *C. guilliermondii* DSM 70052, *C. krusei* DSM 6128, *C. parapsilosis* DSM 5784, *C. tropicalis* DSM 70151, *Rhodotorula glutinis* DSM 70398 and *Yarrowia lipolytica* DSM 70561 were used, purchased from the German collection of microorganisms and cell cultures (DSMZ). Quorum-quenching was assessed using *P. putida* F117 (pKR-C12) a strain reporter for the las QS-system of *P. aeruginosa*, responding to long chain HSLs (Steidle et al., 2001) - and *E. coli* MT102 (pSB403) - a strain reporter for *lux* genes of *Vibrio fischeri*, responding to short chain HSLs (Winson et al., 1998), both kindly provided by Prof. Katharina Riedel. Rhamnolipid B and pyocyanin biosyntheses were evaluated employing *P. aeruginosa* PA14.

Isolation and identification of fungi

Coprinus comatus was collected in Braunschweig, Germany, and isolated on Potato Dextrose Agar (PDA): small tissue fragments from the inner part of the fruiting bodies were excised and placed on PDA. After mycelial development, DNA from fungal colonies

was extracted using the NucleoSpin® PlantII kit (Macherey-Nagel). Fungal primers ITS1F and ITS4 were used for PCR amplification (Bruns *et al.*, 1990); amplicons were sequenced on an Applied Biosystems 377 genetic analyser and analyzed with Sequencher 4.10.1.

Fermentations

From Petri dishes with yeast and malt extract (YEM) agar at 22°C, 5 x 5 mm pellets of *C. comatus* mycelium were transferred to 100 ml Erlenmeyer flasks containing 35 ml of either biotin-aneurin-folic acid (BAF), potato dextrose (PD) or YEM broth, at pH 5.0, pH 7.0 or pH 9.0, respectively. After 14, 21, 28, 35 and 42 days of static incubation at 20°C or 25°C in the dark, the broth was filtrated, mycelia obtained from cultivation, as well as its fruiting bodies were macerated and treated with PBS buffer pH 7.0. After 1 h, pH was adjusted to 4.0, supernatants were extracted with EtOAc and tested for antimicrobial and antibiofilm activities.

Antimicrobial activity and inhibition of biofilm formation

The microdilution method was used for the determination of minimal inhibitory concentrations (MIC).

Bacteria were incubated in LB medium, at 37°C, containing serial diluted coprinuslactone, using a concentration range from 600 µg mL⁻¹ to 15 µg mL⁻¹. Bacterial growth was evaluated over 20 h, employing one OD₆₀₀ measurement each hour in a Bioscreen-C automated growth curve analysis system. Sterile LB medium was set as background. Experiments were performed in duplicate. To evaluate antibiotic or bacteriostatic effects, 10 µL culture aliquot was diluted in 90 µL of fresh LB broth and

plated in LB agar plates after OD measurements. Plates were incubated at 37°C during 24 h. Yeasts were incubated in RPMi (Sigma Aldrich) at 37°C for 24 to 48 h using the same coprinuslactone incubation range (Radetsky *et al.*, 1986).

S. aureus DSM 1104 and *P. aeruginosa* PA14 were used in biofilm inhibition assays in 96-well plates (Falcon® Micro Test™). After overnight incubation at 37°C, bacteria were inoculated in CASO broth with the active compound (300 µg mL⁻¹ to 0.3 µg mL⁻¹, MeOH as control). Biofilms were quantified with crystal violet (Meritt *et al.*, 2005).

Purification and identification of active compounds

Fractionation of the active extracts was carried out in a gradient with an Agilent HPLC 1200 series system (C18 analytical column Nucleosil® 125 mm x 20 mm). Solvent A: acetonitrile, solvent B: Milli-Q® water, with the gradient from 5% A at 0 min to 100% A at 15 min; UV absorbance was detected at 210, 230, 254, 260 and 400 nm; flow rate: 0.5 mL min⁻¹. Fractions were collected every 15 s in a microtiter plate, dried (N₂ stream) and then antimicrobial and biofilm inhibition tests using *P. aeruginosa* PA14 and *S. aureus* DSM 1104 were performed. The active compound was purified with the same method using a C18 column (Varian, Agilent Technologies 250 x 10 mm). Structures were elucidated by ¹H- and ¹³C-NMR including ¹H-¹H- and ¹H-¹³C-COSY, recorded on a Bruker 600 MHz spectrometer, in CD₃OD in a 5 mm tube, TMS was i. standard.

Assessment of bacterial biofilms architecture

Overnight cultures of *P. aeruginosa* PA14 and *S. aureus* DSM 1104 were adjusted to 1x10⁸ CFU mL⁻¹ in CASO broth and 200 µL aliquots/well were added to an 8-well glass chamber system for *S. aureus* DSM 1104 and an 8-well Permanox chamber (Nunc,

Thermo Fisher Scientific) for *P. aeruginosa* PA14. Chamber systems were incubated for 16 h for bacterial attachment and then washed with PBS to remove planktonic cells. Fresh CASO broth containing two-fold serial dilutions of the active compound, starting from 300 $\mu\text{g mL}^{-1}$ for *P. aeruginosa* PA14 and from 150 $\mu\text{g mL}^{-1}$ for *S. aureus* DSM 1104, were added in a final volume of 200 μL and incubated (37°C, 18-20 h, 10 μL of MeOH as control). Then, wells were washed with PBS, stained with Live/dead® BacLight™ bacterial viability kit (Invitrogen™) and finally examined under a Leica TCS SP1 confocal laser scanning microscope with 63 x 0.9 NA lenses (laser lines 488 nm and 561 nm). The thickness of *P. aeruginosa* PA14 biofilms were determined using Z-stacking, where the Z values are set for the beginning and end of a sample. Damage of the biofilm was calculated according to the area covered by green or red cells (softwares ImageJ and Fiji). Imaris was used for biofilm 3D reconstruction.

Quorum sensing inhibition

Strains F117 and MT102 were incubated overnight, 30°C, in LB broth with ampicillin or tetracycline. Cell suspensions (5 ml) were added to 20 mL of fresh media containing the homoserine lactones (HSLs), 50 nM 3-oxo-C₁₂-HSL for F117 and 100 nM of 3-oxo-C₆-HSL for MT102. One hundred μL were added with the active compound in two-fold serial dilutions, ranging from 250 $\mu\text{g mL}^{-1}$ to 0.025 $\mu\text{g mL}^{-1}$, to the wells of a black microtiter plate. Background control was fresh inoculated media without HSLs, MeOH was used as control. After 4 and 8 h of incubation fluorescence or bioluminescence was measured. QS inhibition was calculated relative to the control.

Pyocyanin and rhamnolipid B quantification

P. aeruginosa PA14 (1×10^8 cells) was inoculated in LB media plus two-fold serial dilutions of the active compound ($300 \mu\text{g mL}^{-1}$ to $0.3 \mu\text{g mL}^{-1}$) in a microtiter plate. MeOH was used as control. After 30 h of incubation at 37°C , 1 mL of media were extracted with EtOAc. Pyocyanin and rhamnolipid B were quantified in triplicate using HPLC - MS/MS (Agilent 1200 series system, C18 analytical column (Nucleosil® 250 x 20 mm, Macherey-Nagel). A 6460 TripleQuad with electrospray ionization (ESI) was used for mass spectrometry, under the following conditions: Solvent A: acetonitrile/formic acid (0.1 %), solvent B: Milli-Q®water/formic acid (0.1 %); gradient from 95% B at 0 min to 0% B at 15 min; flow rate: 0.8 mL min^{-1} ; UV absorbance was detected at 210, 230, 254, 260 and 400 nm. Ionization was performed in the positive mode, N_2 flow 9 L min^{-1} . The target parent ions (211 m/z $[\text{M} + \text{H}]^+$ for pyocyanin and 652 m/z $[\text{M} + \text{H}]^+$ for rhamnolipid B) were selected and fragmented (30 V collision energy). The two most intense product ions (pyocyanin: 168 m/z and 77 m/z ; rhamnolipid B: 217 m/z and 70 m/z) were monitored in multiple reaction monitoring (MRM) mode. Compound concentrations were calculated by the Quantitative Analysis MassHunter software (Agilent Technologies) using a pyocyanin commercial standard. Rhamnolipid B concentration was calculated relative to the control.

Transmission electron microscopy (TEM)

P. aeruginosa PA14 and *S. aureus* DSM 1104 were cultured in LB broth overnight at 37°C , centrifuged, cell pellets were washed with PBS and resuspended in fresh media containing the MIC of the active compound, diluted in $5 \mu\text{L}$ MeOH (controls only MeOH). After overnight incubation, cells were washed with PBS, fixed, stained (Yakimov *et al.*,

1998) and examined using a Libra120plus (Zeiss) TEM microscope at 120 kV acceleration voltage in the elastic brightfield mode (10 eV energy-slit width).

MurA expression - MurA inhibition assays

Plasmid pQE30TEV-120.1s, coding for a TEV (Tobacco Etch Virus) protease recognition site C-terminal of a His₆-Tag, was produced by PCR using vector pQE30 (Qiagen) as template (Gulotta, 2015). Endonuclease digestion of plasmids pQE30TEV-120.1s (25 μ L) was achieved by using 1 μ L FastDigest™ enzyme *Bam*HI and *Eco*RV and 5 μ L FastDigest™ Buffer (Thermo Scientific), in 50 μ L for 1 h at 37°C. The digested plasmid was isolated and purified with NucleoSpin® Gel and PCR Clean-up Kit (Macherey-Nagel), following manufacturer's instructions. The plasmid was analyzed by agarose gel electrophoresis (1% wt/vol agarose), stained with ethidium bromide and visualized using a Kodak Image Station 2000 (Carestream Health).

Coding regions corresponding to the mature full length of *P. aeruginosa* PA14 Mur A proteins were amplified by PCR using specific primers (murA_FW GCGGATCCATGGATAAACTGATTATTACCGGC and murA_Rev GCGATATCTTACTAGCCCGGTACGCGGCG). An initial denaturation (98°C, 30 s) was followed by 25 cycles of denaturation (98°C, 10 s), annealing (55°C, 30 s) and amplicon specific elongation (72°C, 60 s). Final elongation was carried out at 72°C for 5 min. The PCR product was cloned between the *Bam*HI and *Eco*RV cleavage site of the pQE30TEV-120.1s vector. For overexpression of the Mur A protein *E. coli* M15 pRep4 (Qiagen) was transformed with the produced plasmids and cultured in Luria-Bertani medium supplemented with ampicillin 100 μ g mL⁻¹ and kanamycin 25 μ g mL⁻¹ to an OD₆₀₀ of 0.5. Then, protein expression was induced by adding 1 mM isopropyl β -D-

thiogalactopyranoside and the cells were cultivated overnight at 30 °C. Bacteria were harvested by centrifugation (15 min; 18.000 × g), resuspended in LEW Buffer (Macherey-Nagel) containing protease inhibitor (cOmplete, Mini, EDTA-free, Roche) and homogenized using a French Press at 1000 psi. After centrifugation (20 min, 31.000 × g) proteins were isolated from the lysate by affinity chromatography using Protino Ni-TED 2000 packed columns (Macherey-Nagel) following manufacturer's protocol.

Mur A was incubated together with the active compound, in a concentration range of 80 – 2.5 µg mL⁻¹. Enzyme inhibition was measured using Lanzetta reagent (Lanzetta *et al.*, 1979), as described previously (Baum *et al.*, 2001).

Cytotoxicity assays

Mouse fibroblast L-929 cells (DSMZ ACC2) were challenged with the active compound, added in two-fold serial dilutions, starting with with 30 µg mL⁻¹. Cell viability was assessed by MTT assay (Berridge *et al.*, 2005). Cell morphology was evaluated using α-tubulin and DAPI staining, where cells were challenged with 30 µg mL⁻¹ of the active compound and visualized under a fluorescent microscope, described in detail by Schneider *et al.*, 2012.

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Conflict of Interest

The authors declare no conflict of interest.

Supplementary information is available at the journal website.

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Titles and legends to figures

Figure 1 (A) HMBC (Heteronuclear Multiple Bond Correlation) spectrum from coprinuslactone **1**; (B) ^1H and ^{13}C NMR data (d_4 -methanol) of coprinuslactone **1** and dihydro-coprinuslactone **2**. In brackets: coupling constants in Hz and (C) chemical structures of: **1** - (3R,4S)-2-methylene-3,4-dihydroxypentanoic acid 1,4-lactone (coprinuslactone); **2** - (2S,3R,4S)-2-methyl-3,4-dihydroxypentanoic acid 1,4-lactone; **3** - 2-methyl-pentene-olide, isolated from *C. comatus*; **4** - (3S,4R)-3-carboxy-2-methylene-heptan-4-olide, isolated from *L. theobromae*; **5**- tulipalins A and **6** tulipalins B, isolated from *T. gesneriana*.

Figure 2 Growth curve of *Pseudomonas aeruginosa* PA14 under different concentrations of coprinuslactone. Triangles: untreated culture, rhombs: $75\ \mu\text{g ml}^{-1}$ coprinuslactone, squares: $150\ \mu\text{g ml}^{-1}$ coprinuslactone. While $150\ \mu\text{g ml}^{-1}$ coprinuslactone are bacteriostatic half of the concentration had no effect on growth.

Figure 3 Effects of different concentrations of coprinuslactone **1** on *S.aureus* (A-D) and *P. aeruginosa* (E-H) *in vitro* biofilms and on L-929 mouse fibroblast cells (I, K). Biofilms were stained using the Live/Dead[®] staining kit and visualized under a confocal laser scanning microscope. (A) *S. aureus* biofilm treated with MeOH (control); (B) treatment with isopropanol (negative control); (C) treatment with $50\ \mu\text{g mL}^{-1}$ and (D) treatment with $100\ \mu\text{g mL}^{-1}$ of coprinuslactone **1**. (E) *P. aeruginosa* biofilm treated with MeOH (positive control); (F) treatment with $37.5\ \mu\text{g mL}^{-1}$ of coprinuslactone **1**; (G) treatment with $75\ \mu\text{g mL}^{-1}$ and (H) treatment with $150\ \mu\text{g mL}^{-1}$ of coprinuslactone. L-929

mouse fibroblast cells immunofluorescence images, stained with anti- α -tubulin and DAPI. (I) cells treated with MeOH, the control; (K) cells treated with 30 $\mu\text{g mL}^{-1}$ of coprinuslactone **1**. Multinucleated and normal cells can be discerned.

figure 1

figure 2

figure 3