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Antioomycete Activity of γ-Oxocrotonate Fatty Acids against *P. infestans*

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Infections with *Phytophthora infestans*, the causal agent of potato and tomato late blight disease, are difficult to control and can lead to considerable agricultural losses. Thus, the development of new effective agents against the pathogen is of great interest. In previous work, (*E*)-4-oxohexadec-2-enoic acid (**3**) was isolated from *Hygrophorus eburneus*, which exhibited fungicidal activity against *Cladosporium cucumerinum*. Here, the inhibitory effect of **3** on *P. infestans* spore germination and mycelium growth in vitro is demonstrated. The in vivo effect on infections of whole potato plants was investigated by spraying plants with the sodium salt of **3**, sodium (2*E*)-4-oxohexadec-2-enoic acid (**4**), prior to *P. infestans* inoculation. Additionally, the influence of **3** on mycelium growth of *Colletotrichum coccodes*, the causal agent of potato black dot disease, was analyzed. In all approaches, a significant inhibition of pathogen development was achieved. Importantly, the unsaturated fatty acid exerted no toxic effect when sprayed on plants, a prerequisite for its commercial use.

KEYWORDS: γ-oxocrotonate fatty acid; *Phytophthora infestans*; oomycetes; fungicides; *Hygrophorus*; synthesis

INTRODUCTION

Phytophthora infestans (Mont.) de Bary, the causal agent of potato and tomato late blight disease, is known as one of the most destructive pathogens worldwide. Infections can lead to total losses, due to the remarkably rapid completion of the pathogen's life cycle within a few days (*1*).

Phylogenetically, *P. infestans* belongs to the Peronosporomycetes (Oomycetes), which traditionally were placed in the kingdom Fungi. However, as a result of molecular and biochemical approaches, the Peronosporomycetes are now classified in the phylum Stramenopiles (Heterokontophyta) (*2*−*4*). This can serve as an explanation for the ineffectivity of most conventional fungicides against *P. infestans*, because typical fungal target sites of fungicide action are absent in Peronosporomycetes (*5*).

Until the beginning of the 1980s, Metalaxyl was the most widely used conventional fungicide to control *P. infestans*. It is a single-target site fungicide and exerts its function by binding irreversibly to the oomycete RNA polymerase-I-template complex, thereby inhibiting rRNA synthesis (*5*). The first Metalaxyl-resistant strains of *P. infestans* appeared in the early 1980s in Europe, for example, in The Netherlands (*6*) and Ireland (*7*). In the following years, resistant populations spread all over the world, reaching the Far East in the mid 1980s (*8*) and the United States in the early 1990s (*9*, *10*). These new populations exhibited a stronger aggressiveness and replaced the "old" ones in many areas within only 5−7 years (*11*). Because both mating types of *P. infestans* (A1 and A2) can nowadays be found all over the world and because worldwide migrations of certain populations were verified, sexual reproduction is possibly responsible for changes in the population characteristics and the appearance of new resistances against other fungicides (*9*, *12*) as discussed, for example, for resistance against the new fungicide Zoxamide (*13*).

Nevertheless, research on safe compounds with antioomycete activity is still of great interest, because effective agents are very limited. In recent years, for example, the carboxylic acid amide fungicides (CAA) were shown to exert anti-*P. infestans* activity (*14*, *15*), as well as bikaverin and fusaric acid (*16*), thiobutacin (*17*), and ethaboxam (*18*).

Another pathogen of potato, the ascomycete *Colletotrichum coccodes* (Wallr.) S. Hughes (the causal agent of black dot disease), which infects all underground parts, has always been regarded as a minor pathogen, only able to infect weakened plants (*19*). Occasionally, tuber infections with *C. coccodes* were confused with *Helminthosporium solani* Durieu and Mont. infections due to similar symptoms (*20*). In the last years, *C. coccodes* has received more attention due to the fact that there is a strong increase in the demand for tubers of excellent quality and appearance (*19*). Indeed, tuber skin blemish diseases caused by *C. coccodes*, but also by *H. solani* and *Rhizoctonia solani* J. G. Kühn, are responsible for great losses due to reduced quality and, as a consequence, downgrading of potatoes destined for consumer markets (*20*).

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Fruiting bodies of the basidiomyceteous genus *Hygrophorus* (Tricholomataceae) are only rarely attacked by parasites. This suggests that these fungi produce secondary metabolites with biocidal activity. In previous studies, we described the isolation and structure elucidation of antibiotic cyclopentenones (hygrophorones) and unusual fatty acids with γ-oxocrotonate partial structure. Both types of compounds revealed fungicidal activity against the ascomycete *Cladosporium cucumerinum* Ell. et Arth. (*21*, *22*). Based on these initial findings, the most promising candidates were synthesized for further activity studies. In this paper, we present results of in vitro and in planta studies concerning the inhibitory effects of sodium (*E*)-4-oxohexadec-2-enoic acid (**4**) on *P. infestans* spore germination, mycelium growth, and infections of potato plants. Additionally, we performed in vitro experiments with *C. coccodes* to test for inhibitory effects on mycelial growth. Our identification of γ-oxocrotonate as an antioomycete compound might enable the development of novel agents against plant diseases caused by *Phytophthora* spp.

MATERIALS AND METHODS

General. All reagents were reagent grade and used without pretreatment, and solvents were purified and dried by standard methods. Melting points were determined by standard methods on a melting point apparatus (DM LS2 Leica) and were uncorrected. Reactions were monitored by TLC on silica gel (Kieselgel 60 F_{254}, 0.040−0.063 mm, Merck) with detection either by UV light or molybdato phosphoric acid. Solutions were concentrated under reduced pressure at 40 °C. Column chromatography was performed on silica gel 60 (0.063−0.200 mm, Merck). ^{1}H (300 or 400 MHz) and ^{13}C (75.5 or 100.5 MHz) NMR spectra were recorded at room temperature on VARIAN Mercury spectrometers. Chemical shifts (**1**, **2**) were referenced to internal TMS (δ = 0 ppm, ^{1}H) and $CDCl_3$ (δ = 77.0 ppm, ^{13}C), respectively. For compound **3**, deuterated ethanol was used as solvent; chemical shifts were referenced to internal solvent methyl group signals (δ = 1.11 ppm, ^{1}H, and δ = 17.2 ppm, ^{13}C). The positive and negative ESI and APCI mass spectra were obtained from an API 150Ex (Applied Biosystems) equipped with a turbo-ion-source. The high resolution positive and negative ion ESI mass spectra were obtained from a Bruker Apex 70e Fourier transform ion cyclotron resonance mass spectrometer (Bruker Daltonics) equipped with an Infinity cell, a 7.0 T superconducting magnet (Bruker), a RF-only hexapole ion guide, and an external electrospray ion source (Agilent). *P. infestans* was cultivated on oat-bean-medium (3.4% [w/v] bean flour, 1.7% [w/v] oat flour, 0.85% [w/v] sucrose, and 1.5% [w/v] Bacto-Agar, 5 μg/mL Geneticin). For measuring the GFP-emitted light, a Cytofluor II Platereader (Millipore Corp.; excitation 485 nm, emission 530 nm) was used. GFP-fluorescence pictures were taken with a Leica MZ FLIII Fluorescence-Stereomicroscope (Leica Microsystems). Realtime PCR measurements were carried out as described (*23*). For measuring the increase in *C. coccodes* biomass a MRX Plate Reader 1.12 (Dynatech Laboratories) was used.

Reagents and Solvents. Tetrahydrofuran (THF), *n*-butyllithium (*n*-BuLi), furan, sodium hydroxide, HCl, pyridine, *N*-bromo-succinimid (NBS), 2-methyl-2-butene, $NaClO_2$, and dodecylbromide were purchased from usual laboratory providers.

Synthesis of Compounds. 2-Dodecylfuran (1). To an ice-cold solution of furan (5.34 mL, 73.5 mmol) in THF (100 mL) at 0 °C, *n*-BuLi (27.3 mL, 2.7 M in hexane, 73.5 mmol) was added dropwise under stirring. After 1 h at 0 − 5 °C, the solution was cooled to − 40 °C and was stirred for further 20 min. Then, dodecylbromide (17.6 mL) in THF (20 mL) was added. The mixture was allowed to warm to room temperature and was stirred for another 5 h. The reaction was quenched with saturated aqueous $NaHCO_3$ solution (20 mL) and the solution was extracted twice with ethyl acetate (2 × 50 mL). The combined organic layers were dried over Na_2SO_4 and concentrated to give a yellow oil, which was purified by column chromatography (dichloromethane) to afford the desired product **1** (13.8 g, 57.9 mmol, 80%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.88 (t, 3H, J = 6.7 Hz, H-16), 1.20−1.40 (m, 18H), 1.56−1.69 (m, 2H), 2.60 (t, 2H, J = 7.6 Hz, H-5), 5.96 (m, 1H, H-3), 6.26 (dd, 1H, J = 3.3, 1.9 Hz, H-1), 7.28 (dd, 1H, J = 1.7, 0.8 Hz, H-2); ^{13}C NMR (75.5 MHz, $CDCl_3$) δ 14.2 (C-16), 22.8, 28.0, 28.1, 29.3, 29.4, 29.5, 29.6, 29.6, 29.7, 29.8, 32.0 (C-5), 104.4 (C-3), 109.9 (C-2), 140.5 (C-1), 156.5 (C-4); (+)-APCI-CID-MS: 237 $[M + H]^+$.

(2*E*)-4-Oxohexadec-2-enal (2). To a mixture of **1** (1.00 g, 4.24 mmol) and $NaHCO_3$ (712 mg, 8.48 mmol) in acetone/H_2O (10:1, 20 mL), NBS (905 mg, 5.11 mmol) dissolved in acetone−H_2O (10 mL) was added at −20 °C. After 1 h stirring at −20 °C, pyridine (0.69 mL, 8.48 mmol) was added. Then the reaction mixture was allowed to warm up to room temperature and stirred for further 2 h. The solution was washed with 1 N HCl, followed by extraction with ethyl acetate (2 × 50 mL). The organic layer was dried over Na_2SO_4 and concentrated to obtain the crude product, which was purified by column chromatography (solvent dichloromethane) to afford the product as a slightly yellow oil (642 mg, 2.55 mmol, 60%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.88 (t, 3H, J = 6.7 Hz, H-16), 1.19−1.36 (m, 18H), 1.59−1.71 (m, H-5), 2.69 (t, 2H, J = 7.8 Hz, H-5), 6.73−6.92 (m, 2H, H-2,3), 9.78 (d,CHO, J = 7.0 Hz, H-1); ^{13}C NMR (75.5 MHz, $CDCl_3$) δ 14.2 (C-16), 22.8, 23.7, 29.2, 29.3, 29.4, 29.5, 29.5, 29.6, 29.7, 32.0, 41.3 (C-5), 137.2 (C-2), 144.8 (C-3), 193.2 (C-1), 199.9 (C-4); (−)-ESI-CID-MS: 251 $[M - H]^-$; ESI-FT-ICR-MS: m/z 251.20137 (calcd for $C_{16}H_{27}O_2^-$, m/z 251.20165).

(2*E*)-4-Oxohexadec-2-enoic Acid (3). To a solution of aldehyde **2** (400 mg, 1.58 mmol) and 2-methyl-2-butene (1.69 mL, 15.8 mmol) in *t*-BuOH (20 mL), NaH_2PO_4 (2.00 g, 16.7 mmol) and $NaClO_2$ (181 mg, purity 80%, 1.89 mmol), both dissolved in H_2O (10 mL), were added, and the resulting mixture was stirred for 2 h at room temperature. Most of the solvent volume was removed under reduced pressure. Ethyl acetate (50 mL) and brine (10 mL) were added to the residue. The aqueous layer was acidified to pH = 1 by a dropwise addition of 1 N HCl. The organic layer was separated, and the aqueous layer was extracted with ethyl acetate (2 × 50 mL). The combined organic layers were dried over Na_2SO_4 and concentrated under reduced pressure to afford the product **3** as a pale yellow solid (350 mg, 1.31 mmol, 83%), mp 98 ± 0.5 °C. ^{1}H NMR (400 MHz, CD_3CD_2OD) δ 0.87 (t, 3H, J = 7.0 Hz, H-16), 1.08−1.34 (m, 18H), 1.56−1.63 (m, 2H), 2.68 (t, 2H, J = 7.0 Hz, H-5), 6.65 (d, 1H, J = 16.2 Hz, H-2), 7.01 (d, 1H, J = 16.2 Hz, H-3); ^{13}C NMR (100.5 MHz, CD_3CD_2OD) δ 15.5 (C-16), 24.6, 25.7, 26.2, 31.0, 31.3, 31.4, 31.5, 31.6, 31.6, 33.9, 43.0 (C-5), 133.7 (C-2), 141.3 (C-3), 169.4 (C-1), 202.8 (C-4); (−)-ESI-CID-MS: m/z 267 $[M - H]^-$, 535 $[2M\text{-}H]^-$; (−)-ESI-CID-MS: m/z 269 $[M - H]$; ESI-FT-ICR-MS: m/z 267.19633 (calcd for $C_{16}H_{27}O_3^-$, m/z 267.19633).

Sodium (2*E*)-4-Oxohexadec-2-enoate (4). To a solution of acid **3** (115 mg, 0.43 mmol) in THF (100 mL), NaOH (17.1 mg, 0.43 mmol) dissolved in H_2O (5 mL) was added. After 30 min, the pH value was checked and adjusted with NaOH to pH = 7.5. The solvent was removed under reduced pressure to afford a white solid powder **4** (118 mg, 0.41 mmol, 95%).

***P. infestans* (Mont.) de Bary Growth Conditions.** For *P. infestans* experiments, the isolate 208m2, carrying a GFP-construct (*24*), was used (kindly provided by F. Mauch, Fribourg, Switzerland). Solutions of zoospores were prepared by growing *P. infestans* for 11 days on oat-bean medium in the dark (18 °C). The mycelium was flooded with 10 mL of deionized water, kept for 4 h at 4 °C to allow zoospore release, and then filtered through one layer of cheesecloth to remove mycelium and sporangia. The solution was adjusted to 1 × 10^5 spores/mL. Sporangia solutions were prepared by flooding mycelium grown for 11 days with 10 mL of deionized water, immediate strong shaking to break off the sporangia from the sporangiophores, and adjusting the solution to 1 × 10^4 sporangia/mL.

***P. infestans* Bioassays and Infection Experiments.** Spore germination experiments with *P. infestans* zoospore solutions were carried out as described (*25*). Briefly, a dilution series of **3** dissolved in 96% ethanol was applied. The final concentrations in the spore solutions ranged from 10 nM to 100 μM and 2% (v/v) ethanol each. Control treatments with 2% (v/v) ethanol were included in the analyses. After treatment, the spores were kept at 4 °C overnight to allow germination. The percentage of germinated spores was calculated after counting spores on photographs taken from five nonoverlapping areas of a 10 μL droplet on a hemocytometer under a light microscope. Spores were counted as germinated when the germtube was at least as long as the spore diameter.

The effect of **3** on *P. infestans* mycelial growth was tested by measuring the increase of GFP fluorescence over the time according to Prost et al. (*25*). Microtiter plates (24-well, NUNC A/S, Denmark) with oat-bean medium were inoculated with 100 μL of a *P. infestans* sporangia solution

and grown in darkness (17 °C). After 24 h, different concentrations of **3** were added. The final concentrations (calculated for 100 μL of sporangia solution) ranged from 10 nM to 1 mM and 1% (v/v) ethanol. *P. infestans* growth was determined by measuring GFP-emitted light (excitation 485 nm, emission 530 nm).

To investigate the direct influence of **3** on living mycelium of *P. infestans*, three-week-old mycelium was drop-inoculated (10 μL) with different concentrations of **3**. A total of 24 h later, GFP-fluorescence pictures of inoculated areas were taken.

Potato plants (*Solanum tuberosum* L. cv. Désirée) were grown as described (*26*). Prior to inoculation with a *P. infestans* zoospore solution, plants were sprayed with different concentrations of **4** dissolved in water at the abaxial leaf surface until runoff. A total of 2 h later, when sprayed leaves had dried, *P. infestans* was inoculated at the abaxial leaf surface (six 10 μL droplets per leaf; 1×10^5 spores/mL; two leaves per plant). Inoculated leaves were then covered with a plastic bag to provide 100% rel. humidity for spore germination. After 3 days, the inoculation sites were cut out with a cork borer and all leaf discs of a given leaf were combined as one sample. *P. infestans* biomass determinations based on realtime PCR-measurements were carried out as described (*23*) using *P. infestans*-specific primers (*27*).

***C. coccodes* (Wallr.) S. Hughes Bioassay.** *C. coccodes* (CBS 369.75) was grown for 5 days in 50 mL of liquid soy bean medium (*28*) in the dark on a rotary shaker (18 °C). To isolate the spores, the whole culture was centrifuged at 2100*g* for 5 min at 4 °C. The supernatant containing the spores was centrifuged again (10 min, 6500*g*, 4 °C). After discarding the supernatant, the pelleted spores were carefully washed in deionized water, centrifuged as above, and finally, the spore concentration was adjusted to 1×10^5 spores/mL in soy bean medium. For the biotest, 200 μL of this spore solution were pipetted into each of a 96-well plate (NUNC A/S, Denmark). The plates were incubated in a growth chamber for 24 h at 17 °C in the dark to allow germination. Then the test concentrations of **3** were applied as described above with final concentrations of 0.01−100 μM and 2% (v/v) ethanol. The plates were returned to the growth chamber and increases in fungal biomass were determined by means of daily OD_{590} measurements.

RESULTS

Synthesis. A fast and efficient three-step synthesis of unsaturated fatty acids was developed (**Figure 1**). Synthesis of (2*E*)-4-oxohexadec-2-enoic acid (**3**) began with the simple 2-alkylation of furan via deprotonation with *n*-buthyllithium and subsequent reaction with dodecyl bromide to 2-dodecylfuran (**1**). The oxidative ring-opening of the alkylfuran **1** was performed in the presence of $NaHCO_3$ and NBS to generate (2*E*)-4-oxohexadec-2-enal (**2**). The final step was a $NaClO_2$ oxidation of aldehyde **2** to (2*E*)-4-oxohexadec-2-enoic acid (**3**) in the presence of 2-methyl-2-butene as chlorine scavenger and 1 N HCl (pH = 1). The overall yield of the three-step process was 35%. To enhance the water solubility of **3**, as required for spraying whole plants, the sodium salt was formed quantitatively with NaOH.

***P. infestans* Spore Germination Assay.** To assess the effect of compound **3** on *P. infestans* spore germination, different concentrations were adjusted in prepared zoospore solutions, and germination rates were determined 24 h later (**Figure 2**). Even at very low concentrations (10 nM), germination was decreased and was inhibited more than 50% at 100 nM compared to the ethanol control. At 1 μM, less than 10% of the spores germinated and at 3.7 μM, germination was completely abolished. Increasing concentrations of **3** also affected germtube length, as shown in **Figure 2**. In addition, at 100 μM **3**, lyses of the spores could be observed (**Figure 2**f).

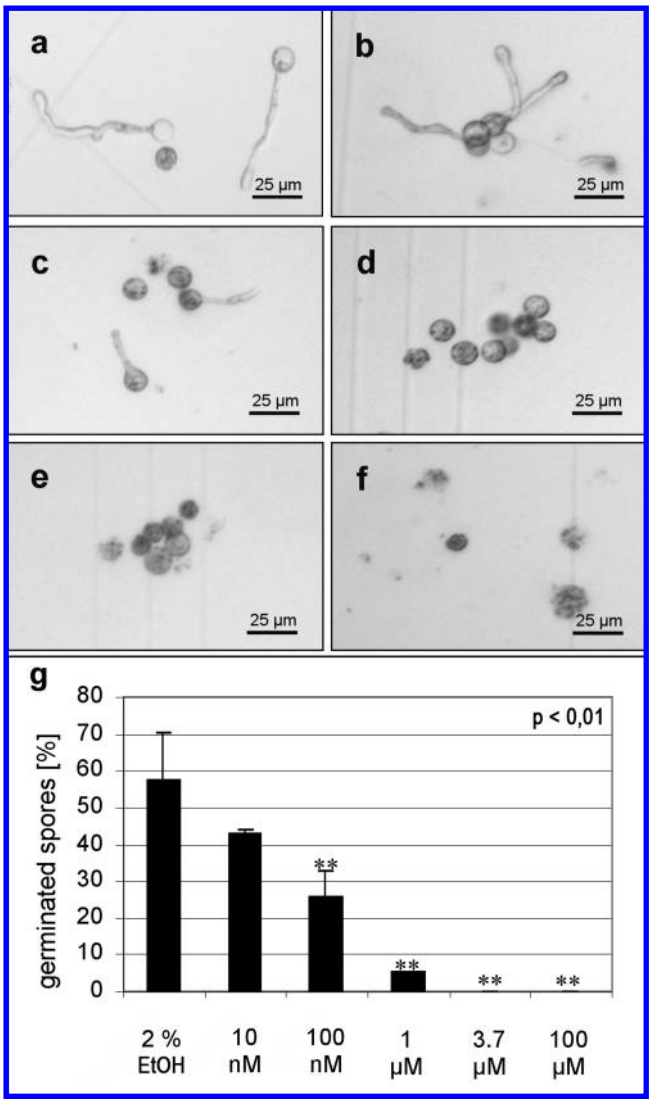

Figure 2. *P. infestans* spore germination is inhibited by compound **3**. *P. infestans* spore suspensions were treated with different dilutions of compound **3**. (a−f) Representative phenotypes of spores in different concentrations of **3**: (a) 2% EtOH, (b) 10 nM, (c) 100 nM, (d) 1 μM, (e) 3.7 μM, (f) 100 μM. Germination rates were calculated after 24 h (g). The diagram shows combined data of two independent experiments (** represent significant differences with $p < 0.01$; one-way ANOVA).

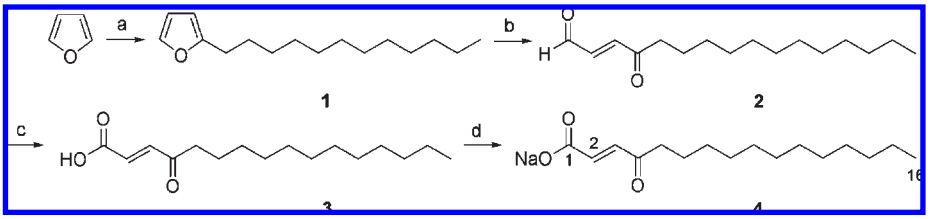

Figure 1. Synthesis of **3**. Reagents and conditions: (a) furan, THF, *n*-BuLi (1.1 equiv) at 0 °C, 30 min, then $C_{12}H_{25}Br$ (1.0 equiv) at −40 °C, warm up to r.t.; (b) **1**, NBS (1.1 equiv), $NaHCO_3$ (2.0 equiv), acetone/ H_2O (10:1), −15 °C, 1 h, pyridine (2.0 equiv); (c) **2**, $NaClO_2$ (1.2 equiv), $Me_2C{=}CHMe$ (10 equiv), *t*-BuOH, H_2O, HCl, 2 h at r.t.; (d) **3**, THF, NaOH (1.0 equiv), r.t., 30 min.

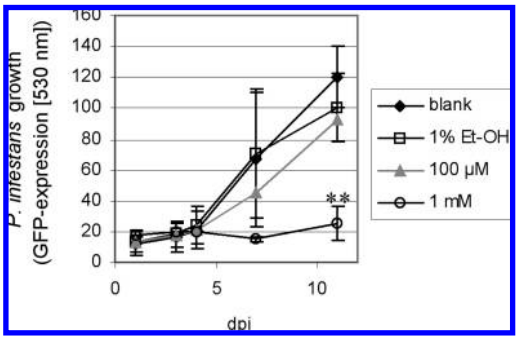

Figure 3. Inhibitory effect of compound **3** on *P. infestans* mycelium growth. One-day-old mycelium was inoculated with **3** in different concentrations. *P. infestans* growth was determined by measuring GFP fluorescence. Graphs show combined data of two independent experiments (** represent significant differences with $p < 0.01$; one-way ANOVA).

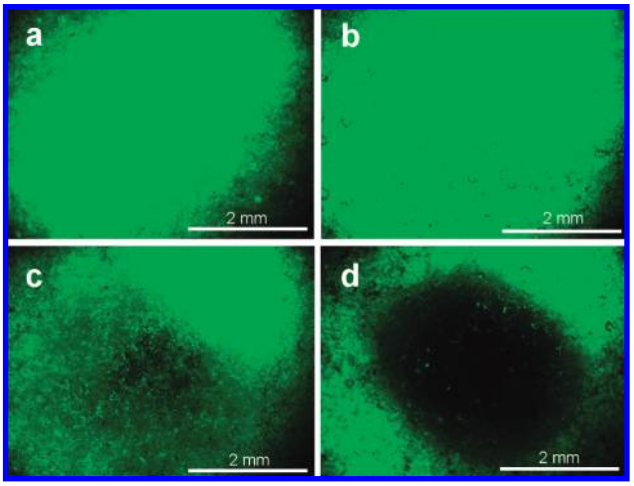

Figure 4. Damaging effect of compound **3** on established *P. infestans* mycelium. *P. infestans* was grown in Petri dishes on oat-bean-agar for 21 days. Then drops (10 μL) of compound **3** in different concentrations were pipetted onto the mycelium. Fungicidal activity led to mycelium damage indicated by a loss of GFP fluorescence. Pictures showing representative treated sites were taken 24 hpi with a fluorescence-stereomicroscope: (a) untreated, (b) 1 μM, (c) 10 μM, (d) 100 μM.

***P. infestans* Mycelial Growth.** The inhibitory effect of compound **3** on mycelial growth of *P. infestans* expressing GFP (*24*) was determined in a bioassay approach (*25*). Different concentrations were applied to mycelium growing in multiwell plates. As a measure for additional mycelial growth, GFP-fluorescence was recorded with a plate reader. **Figure 3** shows that growth was inhibited at high concentrations (1 mM).

We also tested whether oxocrotonate **3** exerts an effect on "mature" mycelium. In this approach, the test solutions were dropped onto three week old mycelium grown on agar plates. Here, also lower concentrations had a clear effect on the viability of the mycelium, as can be seen in **Figure 4**.

***P. infestans* Infections of Pretreated Plants.** The next question addressed was whether pretreating potato plants with compound **4** had an inhibitory effect on the infection with *P. infestans*. For this purpose, plants grown in a phytochamber were sprayed with salt **4** dissolved in water two hours prior to inoculation with a *P. infestans* zoospore solution. The sodium salt of acid **3** had to be used owing to the insolubility of the free acid **3** in water. Three days after the inoculation, pathogen growth determinations were performed, based on a realtime PCR detection method of *P. infestans* DNA (*23*). **Figure 5** shows that pretreating plants

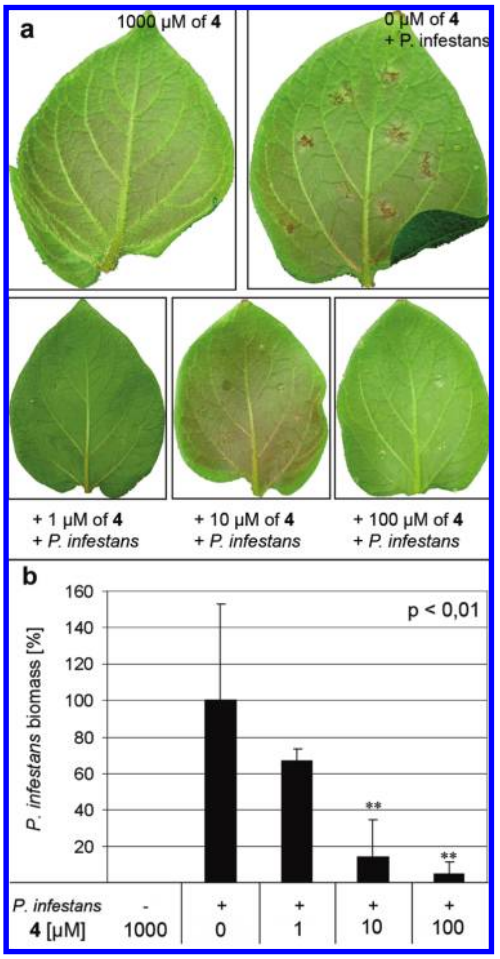

Figure 5. *P. infestans* infections are strongly inhibited by spraying plants with compound **4**. Plants, 21 days old, were sprayed with **4** at the abaxial leaf surface 2 h prior to inoculation with a *P. infestans* zoospore solution: (a) phenotypes of treated leaves, (b) *P. infestans* biomass determinations using infected leaf material (3 dpi). As controls, noninfected samples sprayed with 1000 μM **4** and infected nonsprayed samples were taken (** represent significant differences with $p < 0.01$; one-way ANOVA).

with 10 μM and 100 μM Na-oxocrotonate derivative **4** is sufficient to inhibit the infection with *P. infestans* by about 80 and 95%, respectively, compared to the nonsprayed control. Importantly, spraying the plants at very high concentrations of 1000 μM **4** did not have any toxic effects on the leaves.

Effect of Compound 3 on Mycelium Growth of *C. coccodes*. To determine whether **3** also has an inhibitory effect on the ascomycete *C. coccodes*, a multiwell plate bioassay was established. Based on optical density (OD_{590}) measurements, fungal growth in liquid media was determined. For this purpose, the soy-containing medium developed by Yu et al. was used (*28*). A one-day-old mycelium was inoculated with different concentrations of compound **3** and additional growth of *C. coccodes* was recorded every 24 h. As shown in **Figure 6**, concentrations as low as 0.01 μM **3** significantly inhibited growth of *C. coccodes*. Increasing concentrations further reduced fungal growth. A 1 μM solution of acid **3** restricted growth by more than one-half compared to the ethanol control and 100 μM led to complete inhibition.

DISCUSSION

The class Peronosporomycetes comprises a diverse group of saprophytic and pathogenic species. Among the latter, devastating plant pathogens, but also species infecting animals and

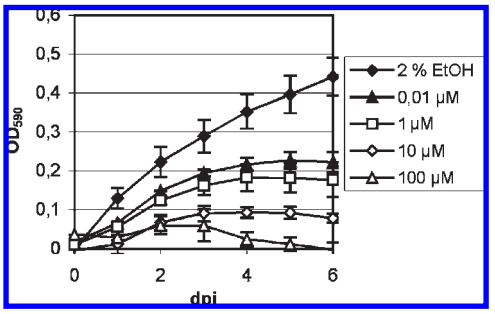

Figure 6. Inhibitory effect of compound **3** on *C. coccodes* mycelium growth. One-day-old mycelium in 96-well plates was inoculated with different concentrations of **3**. Additional *C. coccodes* growth was determined by measuring OD_{590} values. The experiment was performed twice with similar results. Statistical analysis revealed highly significant differences between 2% EtOH treatment and all tested concentrations of **3** at the end of the experiment ($p < 0.01$; one-way ANOVA).

microbes, occur. Besides the genus *Phytophthora*, there are also very important plant pathogens in the genera *Albugo*, *Bremia*, *Plasmopara*, and *Peronospora*. These obligate-pathogenic species are the causal agents of white rust or downy mildew disease on many different plants (*5*). In the genus *Phytophthora*, more than 60 different species mostly infecting dicotyledonous plants were described. Some exhibit a very narrow host range, others are less adapted and can colonize a lot of different plants. Important species apart from *P. infestans* are, for example, *P. sojae* Kaufm. and Gerd. (root rot of soybean), *P. palmivora* E. J. Butler (black pod of cocoa), *P. ramorum* Werres, De Cock and Man in ′t Veld (sudden oak death), and *P. cinnamomi* Rands (dieback and related root rot diseases) (*29*). *P. cinnamomi* displays probably the broadest host range with about 3000 different plant species (*30*). This underlines the great agricultural interest in the identification of new antioomycete substances.

Continuing our research on bioactive secondary metabolites from fruiting bodies of Basidiomycetes, we recently isolated unusual fatty acids with 4-oxocrotonate partial structures from the genus *Hygrophorus*. These natural compounds show antifungal activity against the phytopathogenic fungus *Cladosporium cucumerinum* Ellis and Arth. (*21*, *22*). Based on these initial results, the most promising candidates were synthesized in a very short and efficient way on a multi milligram scale. To provide sufficient amounts for more detailed studies, the synthesis of (2*E*)-4-oxohexadec-2-enoic acid (**3**) was scaled up to produce multigram amounts. Starting materials for the current process are readily available, the process is simple, and if required, altered routes can be devised, too. Thus, no problems are expected for further upscaling.

Compound **3**, as well as its better soluble salt sodium (2*E*)-4-oxohexadec-2-enoate (**4**), exhibited remarkable antioomycete and antifungal properties. For *P. infestans*, the strongest inhibitory effect was observed in the zoospore germination assays where nanomolar concentrations were sufficient to prevent germination. This can be explained by the fact that spore dispersal and germination reflects a critical stage of development, which is dependent on favorable environmental conditions (*31*). Growth of *P. infestans* mycelium was inhibited only at higher concentrations (1 mM), which is indicative of active metabolization and detoxification. In this context, former studies revealed that keto-group-containing oxylipins, which are structurally similar to the α,β-unsaturated carbonyl moiety of acid **3**, are metabolized in planta (*32*) and by oomycetes and fungi (*25*). The doubly activated α,β-unsaturated carbonyl moiety is a reactive Michael acceptor that, for example, easily forms glutathione- and other thiol-conjugates, reactions typical for detoxification processes (*33*). Lower concentrations of compound **3** in the *P. infestans* mycelium growth assay (100 μM) led to slightly delayed growth compared to the controls, which is another hint for active detoxification or metabolization. This could not be seen in old mycelium directly inoculated with **3**. Here, strongly reduced GFP fluorescence suggested a loss of metabolic activity or even hyphal death. Detoxification mechanisms in the mycelium during this late stage of development were possibly not activated due to the fact that asexual reproduction in terms of sporangia formation was already completed. When **4** was sprayed on leaves, efficient protection against subsequent *P. infestans* infection could be achieved. Further studies will address the question whether compound **4** also shows a curative effect when sprayed at different time points after *P. infestans* inoculation. A very important observation was that no toxic effects could be seen on sprayed leaves even at very high concentrations.

In the literature, other naturally occurring fatty acids with γ-oxocrotonate-derived structure were described. From Actinomycetes, (*E*)-4-oxonon-2-enoic acid (*Streptomyces olivaceus* Tü 4018) (*34*) and from Ascomycetes, vermiculine (*Penicillium vermiculatum*) (*35*) were isolated. Both have antibacterial activity. An effective inhibitor of retroviral reverse transcription, (*E*)-4, 5-dioxo-2-decenoic acid, named podoscyphic acid, was isolated from fermentations of an Tasmanian *Podoscypha* species (Basidiomycetes) (*36*). Recently, also fatty acids from another *Hygrophorus* species, *Hygrophorus discoxanthus* (Fr.) Rea, were described (*37*). These compounds are structurally very similar to **3** and the other fatty acids we isolated from *H. eburneus* (Bull.) Fr. (*22*). In a biotest with *Cladosporium cucumerinum*, they also showed inhibition of pathogen growth.

In the natural habitats, we repeatedly observed that fruiting bodies of *Hygrophorus* species are only rarely attacked by mycoparasitic fungi. The only description of a pathogen infestation is reported for *Hygrophorus discoxanthus* (*38*). In this case, colonization of very old fruiting bodies of the macromycete with *Botrytis cinerea* Pers., *Cladosporium cladosporioides* (Fres.) de Vries, and *Penicillium* spec. is described. These species are generalists, feeding on various substrates. Because *Hygrophorus* species are not mentioned as hosts or substrates for mycophilic fungi in literature (*39*), it can be assumed that the variety of unusual fatty acids with a broad sprectrum antibiotic profile, as found in *Hygrophorus*, serves as chemical defense.

Future research will have to show if 4-oxocrotonates can be brought into application. Synthetic accessibility is expected to be unproblematic. The compounds' most dominant chemical features are their amphiphilic properties and the electrophilic Michael-acceptor system. Both can be useful (as active principle) in agrochemicals, and here they might contribute to the high bioactivity. Toxicological evaluations still need to be performed, although the *Hygrophorus* spec. as an original source are not considered as toxic in general.

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