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Control of human and plant fungal pathogens using pentaene macrolide 32, 33-didehydroroflamycoin (DDHR)

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Running headline: DDHR inhibits growth of common fungal pathogens

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Abstract

Aims: The aim of this study is to address the toxicity of recently described polyene macrolide 32, 33- didehydroroflamycoin (DDHR) on a wide range of fungal pathogens and its potential to control plant fungal diseases.

Methods and Results: The antifungal activity of DDHR *in vitro* was examined against common human and plant pathogenic fungi using a broth microdilution assay. MIC concentrations ranged from 12.5 to 35 μg ml^{-1}. A radial growth inhibition assay showed that DDHR inhibited mycelia growth, inducing mycelial necrosis and affecting sporulation. During the *in vivo* assay on apple fruits administration of DDHR 1 hour before fungal inoculation inhibited spreading of the infection. Importantly, DDHR exhibited no phytotoxic effects on the model plant, *Capsicum annum*, verified by the plant growth rate and chlorophyll content.

Conclusions: DDHR inhibits growth of various plant pathogens *in vitro* with the strongest activity against *Alternaria alternata*, *Colletotrichum acutatum* and *Penicillium expansum,* and protects apple fruits from decay.

Significance and Impact of Study: This is the first report of the inhibitory effect of DDHR on important pathogenic fungal isolates. DDHR could be a good scaffold for developing new antifungal agents for fruits and vegetables protection.

Keywords: DDHR, fungi, plant pathogens, polyene macrolide

Introduction

Fungi are causative agents of many animal and plant infections. In humans, fungal pathogens are responsible for various difficult-to-treat superficial or systemic mycoses. In plants they are the main cause of fruit and vegetable spoilage leading to a large reduction in agricultural yields. As broad-host-range pathogens, some fungi can infect numerous plant species and at the same time present a significant threat to immune-compromised individuals indicating both their medical and agricultural importance (Sexton and Howlett, 2006).

Fungal-induced plant diseases are difficult to fight due to pathogens developing resistance to fungicides together with the high toxicity of the chemicals in use (Janisiewicz

and Korsten, 2002, Snelders *et al.*, 2011). In order to reduce the utilization of fungicides, there are constant scientific and technological efforts to develop and apply alternative methods for postharvest fruit protection (Janisiewicz and Korsten, 2002, Moscetti *et al.*, 2013). Despite an increasing number of environmentally friendly technologies, modern agriculture is still heavily dependent on agrochemicals because of their relatively low cost, ease of use, and effectiveness (Schirra *et al.*, 2011). Due to the problem with pesticide resistance and the lack of replacement fungicides, there is a constant demand for novel natural and synthetic compounds with potent antifungal activity and reduced toxicity.

Polyene macrolides are one of the most important subgroups of polyketides, which represents a highly diverse group of natural products (Staunton and Weissman, 2001). To date, more than 200 polyene antibiotics have been discovered, most of them being produced as secondary metabolites by soil *Actinomycetes* belonging to the genus *Streptomyces* (Berdy, 2005). Polyenes such as amphotericin B or nystatin have been known for decades for their potent antifungal activity against important human pathogens (Zotchev, 2003, Caffrey *et al.*, 2008, Stankovic *et al.*, 2013). However, only a few of them have been demonstrated to inhibit the growth of filamentous fungi, which are causative agents of many persistent plant diseases (Cong *et al.*, 2007, Kim *et al.*, 2012, Xiong *et al.*, 2012, Xiong *et al.*, 2013a). The polyene macrolide natamycin is an approved GRAS (Generally Recognized as Safe) agent by the U.S. Food and Drug Administration and is also designated as a natural preservative by the European Union (EEC no. 235). Natamycin has been used for decades as a food additive, protecting against mould formation on the surface of sausages and cheese (Holley, 1981, Fajardo *et al.*, 2010, Pintado *et al.*, 2010). Because of the high antifungal efficiency, but low frequency of resistant pathogen appearance (Joseph-Horne *et al.*, 1996, Ghannoum and Rice, 1999) polyenes may be considered a good scaffold for the development of novel, more efficient fungicides.

32, 33- didehydroroflamycoin (DDHR) was recently described as a polyketide pentaene macrolide (Stodulkova *et al.*, 2011), with strong antifungal activity demonstrated against *Candida albicans* (Stankovic *et al.*, 2013). The aim of this study was to assess the antifungal potential of DDHR on a broad range of pathogenic fungi focusing on species that are responsible for persistent postharvest decay of fruits and vegetables. We examined the activity of DDHR on various human and plant fungal pathogens *in vitro*, and subsequently addressed its protective effect *in vivo* on artificially infected apple fruits.

Materials and Methods

32, 33- didehydroroflamycoin (DDHR) production and purification

DDHR was produced in shake flask culture of *Streptomyces durmitorensis* MS405 strain (DSM 41863; **D**eutsche **S**ammlung von **M**ikroorganismen) (Savic *et al.*, 2007). *Streptomyces durmitorensis* spore suspension (20 µl, corresponding to 2.8 x 10^8 CFU), prepared as earlier described (Kieser *et al.*, 2000), was inoculated into tryptone soy broth (TSB) (tryptone soy broth powder, 30 g l^{-1}) and incubated at 30 ºC on a rotary shaker (200 rpm) for 48 h in the dark. This pre-culture was used for the inoculation (1% inoculum, v/v) of production nutrient extract medium supplemented with mannitol (NEM) containing glucose, 10 g l^{-1}, yeast extract, 20 g l^{-1}, beef extract powder, 1 g l^{-1}, casamino acids, 2 g l^{-1} and mannitol, 20 g l^{-1} (Stankovic *et al.*, 2013). Cultures were grown in the dark at 30 ºC on a rotary shaker (200 rpm) for 7 days and crude ethyl acetate extract containing DDHR was purified using dry flash chromatography, as previously described (Stankovic *et al.*, 2013). Unless otherwise stated, all media components were purchased either from Oxoid (Cambridge, UK), Becton Dickinson (Sparks, MD, USA) or Sigma Aldrich (Munich, Germany). A solution of purified DDHR in dimethyl sulfoxide (DMSO) was used in all assays.

Pathogenic strains and culture conditions

Antifungal activity of DDHR was determined against: (i) Human clinical fungal strains: *Aspergillus flavus* (P-21), *A. fumigatus* (PL-4), *Candida krusei* (ATCC 6258), *Cryptococcus neoformans* (CN35), *Fusarium* sp. (P-27), and *Trichophyton mentagrophytes* (DMT-2) from the collection of National Reference Medical Mycology Laboratory (Institute of Microbiology and Immunology, Faculty of Medicine, University of Belgrade, Belgrade) isolated from patients suffering superficial or systemic mycoses and (ii) phytopathogenic fungal isolates: *Alternaria alternata* (AAJ-2), *A. flavus* (AFJ-5), *Botryosphaeria obtusa* (BOJ-16), *Botrytis cinerea* (BCJ-3), *Colletotrichum acutatum* (CAJ-4), *C. gloeosporioides* (CGJ-7), *Fusarium avenaceum* (FAJ-1), *Monilinia fructigena* (MFJ-2), *Mucor piriformis* (MPJ-7), *Penicillium expansum* (PEJ-5) from the Culture Collection of the Institute for Plant Protection and Environment, Belgrade, all originally obtained from decayed apple fruits. Stock cultures of each pathogen were maintained on potato dextrose agar (PDA) at 4 °C. Working cultures were prepared by transferring a stock agar plug containing mycelium onto PDA in Petri dishes and incubating for 7 days at 25 °C in the dark.

Antifungal activity *in vitro*

The minimum inhibitory concentration (MIC) of DDHR to human fungal pathogenic isolates was determined using a reference method for testing antimicrobial agents for yeasts and moulds (EUCAST, 2008, Rodriquez-Tudela *et al.*, 2008) in a 96-well microtiter plate assay. Briefly, 100 µl of prepared inocula was incubated together with 100 µl of different concentrations of DDHR in RPMI 1640 (Roswell Park Memorial Institute 1640) medium with 2% glucose at 30 °C (for moulds) and 37 °C (for yeasts) and the growth inhibition was assessed over time (24h for *C. krusei*, 48h for *C. neoformans*, *A. flavus*, *A. fumigatus* and *Fusarium* sp, 96h for *T. mentagrophytes*).

The yeast inoculum was prepared by resuspending five colonies in 4 ml of distilled water. Yeast cells suspensions were adjusted to 2.5 x 10^6 CFU ml^{-1} using Neubauer haemocytometer and diluted in RPMI 1640 (2% (w/v) glucose) in a ratio of 1:10 (2.5 x 10^5 CFU ml^{-1}).

The mold inoculums were prepared by resuspending the conidia in 0.9% (w/v) NaCl with 0.1% (v/v) Tween 20 following microscopic examination to ensure that the presence of hyphae is lower than 5% of the fungal structures. After adjusting the suspension to 2.5 x 10^6 CFU ml^{-1}, it was diluted in RPMI 1640 (2% (w/v) glucose) in a ratio of 1:10 (2.5 x 10^5 CFU ml^{-1}). Controls containing solvent were carried out in each assay. MIC was defined as the lowest concentration of compound at which no evident growth was observed. The assay was repeated two times in duplicates.

Standard disc diffusion assays were carried out for the preliminary activity screen of phytopathogenic fungal isolates. Sterile paper discs (HiMedia Laboratories, Mumbai, India) containing 200 µg of DDHR per disc were applied to the PDA plate surface. The same volume of DMSO was used as a control. Plates were incubated at 25 °C for 7 days in the dark and zones of inhibition were measured. The assay was repeated three times, each on a single plate and the values are presented as average of three independent experiments ± standard deviations (SD).

The inhibitory effects on mycelial growth of phytopathogenic fungi were also estimated by using a radial growth inhibition assay. The agar disc of each fungi tested (*A. alternata*, *C. acutatum* and *P. expansum*) was transferred to the surface of the PDA plates, containing different concentrations of DDHR (50, 100, 200 and 300 µg ml^{-1}) and incubated 7 days at 25 °C. Plates containing DMSO and inoculated with test pathogens served as controls. The assay was repeated three times, each on a single plate and the mycelial growth or no growth was recorded.

Antifungal activity *in vivo*

In vivo assays were performed as previously described (Dimkic *et al.*, 2013). Apple fruits (cv. Golden Delicious) were surface sterilized by dipping in ethanol (70%) for 2 min, rinsed twice with distilled sterile water and air-dried. The fruits were wounded (5 mm in diameter and 5 mm in depth from surface) using a cork borer and 50 µl of 200 µg ml^{-1} DDHR, or DMSO in controls, was dropped on the wound by micropipette. After 1 h, the wound was inoculated with 50 µl of each tested fungal conidial suspension (*A. alternata*, *C. acutatum* and *P. expansum*). Conidial suspensions were prepared by flooding the culture plates with 5 ml of sterile distilled water containing 0.1% (v/v) Tween-80 and then gently scraping the agar surface with a glass rod. Mycelial fragments were removed by passing the spore suspensions through double layers of sterile cheesecloth and the spore counts were determined using a haemocytometer, adjusted with sterile distilled water to obtain 10^6 spores per ml. The positive control fruits were inoculated with fungal conidial suspension, while negative controls contained only DMSO. All fruits were placed in a moist chamber and incubated at 25 °C. After 7 days the diameter of necrotic lesions were measured. The percentage of necrosis inhibition (IN) is defined as: IN (%)= KR-R/KR x 100, where KR is radius of necrosis in infected fruit without DDHR treatment and R is radius of necrosis in infected fruit treated with DDHR. The assay was repeated three times in duplicates.

Microscopy

The phytopathogenic fungi tested (*A. alternata*, *C. acutatum* and *P. expansum*) were grown on PDA plates containing 50 µg ml^{-1} DDHR at 28 °C for 7 days. Fungal mycelia were deposited on the surface of microscopic slides containing lactophenol cotton blue. Changes in hyphae morphology were observed using an Olympus BX51 fluorescent microscope supplied with Cytovision 3.1 software (Applied Imaging Corp., San Jose, USA).

Effect of DDHR on plants

Pepper (*Capsicum annuum* L.) seeds were soaked in 1 mM $CaSO_4$ overnight and allowed to germinate between two sheets of filter paper moistened with saturated $CaSO_4$. The 7 day old seedlings were then transferred to a complete nutrient solution (three plants per 2.5 l plastic pot, two pots per treatment) containing (in mmol l^{-1}): 0.7 K_2SO_4, 0.1 KCl, 2.0 $Ca(NO_3)_2$, 0.5 $MgSO_4$, and (in µmol l^{-1}): 0.5 $MnSO_4$, 0.5 $ZnSO_4$, 0.2 $CuSO_4$, 0.01 $(NH_4)_6Mo_7O_{24}$, 10 H_3BO_3, 3 µmol l^{-1} $CoCl_2$, 20 µmol l^{-1} Fe^{III}EDTA (Pavlovic *et al.*, 2013).

Six-leaf plants were treated with 200 µg ml^{-1} DDHR solution by directed foliar spraying. Control plants were sprayed with water containing 1% DMSO. The same treatment was repeated six days after the first application.

Chlorophyll content in the four fully expanded and the two youngest leaves was approximated nondestructively using a portable Chlorophyll Meter SPAD-502 device (Minolta Camera Co., Osaka, Japan) just before application of DDHR and 1, 6, and 7 days following the first treatment with DDHR. Root length and shoot height of each plant were measured before the first application of DDHR and 7 days after first treatment (one day after the second application) and the rooth/shoot ratios were calculated. The assay was repeated three times with six plants per treatment.

Statistical analysis

The results were analyzed by Student's t test using SPSS statistical software. p values <0.05 were considered significant.

Results

***In vitro* antifungal activity of DDHR**

Pure 32, 33- didehydroroflamycoin (DDHR; Fig. 1) was obtained by bacterial fermentation from cultures of *Streptomyces durmitorensis* MS405 in high yields (~110 mg l^{-1}).

We addressed the antifungal activity of DDHR on a wide range of pathogens including clinical isolates. DDHR efficiently inhibited growth of all fungi tested with MIC values between 12.5 and 35 μg ml^{-1} (Table 1).

Since DDHR inhibited growth of *Aspergillus* and *Fusarium* species whose members are also well known plant pathogens, we further tested whether DDHR affected growth of other important postharvest fungal isolates (Table 2). Using a standard disc diffusion assay we demonstrated that DDHR inhibits mycelial growth of most of the fungi tested, with the inhibition zones ranging from 51±3 mm for *C. acutatum* to 13±1 mm for *M. piriformis* after 7 days of exposure (Table 2). Only *B. obtusa* was resistant to DDHR, as no growth inhibition zone was detected.

To examine the efficiency of DDHR on mycelial growth, we selected three fungi that showed high susceptibility to DDHR and cultured them on PDA plates containing increasing concentrations of this compound (Supporting information 1, Fig. S1). The strongest effect was observed for *C. acutatum* where 50 μg ml^{-1} DDHR completely inhibited mycelial growth after 7 days of incubation, while 100 μg ml^{-1} DDHR was sufficient to cause evident growth inhibition of *A. alternata* and *P. expansum*.

Effect of DDHR on fungal hyphae morphology

200 μg DDHR applied on the disc filter paper caused strong necrosis of fungal mycelia in the area of contact with the compound, as demonstrated for *C. acutatum* (Fig. 2 A and 2 B). Examination of *C. acutatum* by microscopy showed extensive sporulation and the regular hyphae shape and size of the mycelia that grew in the absence of DDHR (Fig. 2 C). Presence of 50 μg ml^{-1} DDHR inhibited sporulation and caused changes in hyphal morphology such as hyphal distortion and necrosis (Fig. 2 D). Inhibition of sporulation by DDHR was also observed in *P. expansum*, while changes in hyphal morphology were not as obvious as they were in *C. acutatum* sample (data not shown).

***In vivo* antifungal activity of DDHR**

To address the antifungal potential of DDHR *in vivo* we chose apples as a model system. Following application of 200 µg ml^{-1} DDHR, we infected apple fruits with *A. alternata* (Fig. 3 A and 3 B), *C. acutatum* (Fig. 3 C and 3 D) and *P. expansum* (Fig. 3 E and 3 F) and, examined the potential of DDHR to protect spreading of the disease. DDHR efficiently protected apples from decay and significantly inhibited necrosis ranging from 63.5±0.5 % for *C. acutatum*, 69.5±1% for *A. alternata* to 72.8±1% for *P. expansum* infection ($p<0.0001$, compared to infected fruits not treated with DDHR) (Table 3). Importantly, a protecting effect of DDHR against postharvest fungi was not only observed on the apple peel, but also deep inside tissue (Fig. 3).

Effects of DDHR on plants

As fungi included in this research are not only postharvest pathogens, but also causal agents of many plant diseases, we further tested whether DDHR had harmful effect on plants if used as a foliar spraying agent for crop protection. To address this issue we chose pepper (*Capsicum annuum* L.) which is highly affected by various pathogenic fungi including *Alternaria, Botrytis*, *Colletotrichum, Fusarium,* and *Sclerotinia* species. To determine overall plant condition in response to foliar spraying with DDHR, we measured chlorophyll content, root length and shoot height through the time course of the experiment. Spectral Plant Analysis Diagnostic (SPAD)-index showed the same trend of increasing chlorophyll content in both control (ranging from 27.06 in pre-treatment to 35.04 SPAD units on 7^{th} day) and plants sprayed with 200 µg ml^{-1} DDHR (ranging from 30.77 to 38.07 SPAD units) (Fig. 4 A). Also, root/shoot ratios changed in the same manner in control and treated plants during the experiment (1.98, 1.57 in control plants and 2.06, 1.63 in treated plants in pretreatment and on 7^{th} day, respectively) (Fig. 4 B). Chlorophyll content and root/shoot ratio changes in DDHR-sprayed plants were not statistically significant compared to control plants ($p>0.05$).

Taken together, our results demonstrate that DDHR efficiently protected fungi infected fruits from the spread of disease without any harmful effects to photosynthesis and growth.

Discussion

Polyene macrolides are one of the most important subgroups of polyketides which are well known for their strong antifungal activity. Some of them, such as amphotericin B and nystatin, have been used for decades as treatments for human fungal infections (Zotchev, 2003, Caffrey *et al.*, 2008, Stankovic *et al.*, 2013). DDHR is a secondary metabolite isolated from *Streptomyces durmitorensis* MS405 strain that has recently been described to exhibit toxicity to *Candida albicans* with MIC = 70 μg ml^{-1} (Stankovic *et al.*, 2013). In this study we showed that DDHR potently inhibits growth of a diverse range of fungal pathogenic species including clinical isolates obtained from patients suffering superficial or systemic mycoses. The antifungal activity of DDHR with MIC values between 12.5 and 35 μg ml^{-1} is similar to its recently described analogue PN00053 and filipin, but is more effective than Amphotericin B (Kim *et al.*, 2012, Vartak *et al.*, 2014). Importantly, the toxic effect of DDHR to fungal pathogens is significantly higher than to mammalian cells in culture (IC50 between 50 and 100 μg ml^{-1}) (Stodulkova *et al.*, 2011, Stankovic *et al.*, 2013) suggesting that DDHR may have potential future clinical applications.

Seventy percent of plant diseases are caused by phytopathogenic fungi leading to a large decrease in crop yields (Pan *et al.*, 2010). Although the potential of polyene macrolides for treatments of human infections have been extensively studied, data on the inhibitory effect on the growth of filamentous fungi, causative agents of many persistent plant diseases, remain scarce. Filipin, fungichromin and antifungalmycin 702 have been recently shown to affect plant pathogens (Kim *et al.*, 2012, Xiong *et al.*, 2012, Xiong *et al.*, 2013a). We

demonstrate toxic effects of DDHR to a wide range of plant pathogenic species causing hyphal distortion and necrosis and inhibition of sporulation. Polyene macrolides bind sterols in cell membranes and often have high affinity towards ergosterol. Ergosterol is the major sterol component of the fungal cell membranes and is responsible for maintaining cell function and integrity (Tian *et al.*, 2012). Antifungalmycin 702 inhibits growth of *Rhizoctonia solani* by inducing membrane permeabilization (Xiong *et al.*, 2013b). Filipin also permeabilizes membranes potently inhibiting growth of several plant fungal pathogens (Kim *et al.*, 2012). Natamycin binds ergosterol but performs its antifungal effect without damaging membranes (te Welscher *et al.*, 2008). In our previous report we demonstrated that DDHR killed *C. albicans* cells by damaging membranes and inducing necrosis (Stankovic *et al.*, 2013). Recent results on model lipid membranes showed that DDHR partitions to membranes forming pores whose size and stability depend on the presence of cholesterol (Koukalová *et al.*, 2014). A similar mechanism may be involved in the hyphal necrosis observed here for filamentous fungi. Although polyene macrolide antibiotics commonly interfere with cell membranes, different modes of action are possible, therefore the antifungal mechanism of DDHR remains to be determined.

Apples are common subjects of fungal diseases. *Alternaria alternata*, *Botrytis cinerea* and *Penicillium expansum* are the main postharvest pathogens of apples, causing black, grey and blue mould, respectively (Zhang *et al.*, 2010). *A. alternata* together with *C. acutatum* exhibited the highest sensitivity to DDHR treatment in the disc diffusion assay, followed by *B. cinerea* and *P. expansum* with similar growth inhibition. In addition, DDHR efficienly reduced *A. alternata*, *P. expansum* and *C. acutatum*- induced apples' decay. The protecting effect was observed not only on the apple peel but also deep inside the tissue. Fungal resistance to polyenes is rare, but some naturally resistant cells can occur due to the production of modified sterols, which lowers binding affinity of polyenes and therefore

reduces their detrimental effect on fungal membranes (Vandeputte *et al.*, 2012). In addition, ergosterol masked by metabolites produced on the membrane surface could be less accessible or even inaccessible for polyenes, thus leading to fungal lower sensitivity or even resistance. These mechanisms could explain different susceptibility of fungal pathogens to DDHR, as well as the total lack of growth inhibition observed for *B. obtusa*.

Besides their pathogen-protecting effects on crops, many widely used fungicides can affect the normal physiological condition of plants influencing CO_2 assimilation, photosynthesis, nutrient composition, antioxidative enzymes or their secondary metabolism (Muthukumarasamy and Panneerselvam, 1997, Wu and Von, 2002, Saladin *et al.*, 2003, Nason *et al.*, 2007, Petit *et al.*, 2008). Foliar spraying of DDHR in concentrations that were effective *in vitro* and *in vivo*, did not impair plant growth rate or the synthesis of photosynthetic pigments. Polyene macrolides can interfere with sterols from plasma and outer chloroplast membrane of algae and higher plants, as shown for filipin (Moeller and Mudd, 1982). Such interactions of filipin and sterol have not been detected in the inner chloroplast membrane, which may also explain the lack of interference of DDHR with chlorophyll content observed in this study. Notably, even multiple applications of DDHR did not cause negative effects on overall plant health, indicating its suitability to be used as a foliar spray agent against fungal pathogens.

In conclusion, we report that pentaene macrolide DDHR exhibits inhibitory effects on common human and plant fungal pathogens *in vitro* and protects infected apple fruits *in vivo.* Potent antifungal activity, low cytotoxicity to mammalian cells and the absence of phytotoxic effects, makes DDHR a promising antifungal agent that could be used for protection of fruits and vegetables.

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Conflict of interest:

The authors declare that they have no conflict of interest.

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Tables

Table 1 Antifungal activity of 32, 33- didehydroroflamycoin (DDHR) on clinical fungal isolates after 2 days incubation in 96-well assay

Pathogen	**Origin of the strain**	**MIC ($\mu g\ ml^{-1}$)[a]**
Aspergillus flavus (P-21)	Skin	25
Aspergillus fumigatus (PL-4)	Skin	12.5
Candida krusei (ATCC6258)	ATCC	35
Cryptococcus neoformans (CN35)	Cerebrospinal fluid	12.5
Fusarium sp. (P-27)	Blood	25
Trichophyton mentagrophytes (DMT-2)	Skin	12.5

[a] The minimum inhibitory concentration (MIC) determined as the lowest concentration of compound at which no evident growth was observed. Values are average of two independent experiments performed in duplicates.

Table 2 Antifungal activity of DDHR on phytopathogen fungi after 7 days of exposure in the disc diffusion assay

Pathogen	Inhibition R (mm)[a]
Alternaria alternata (AAJ-2)	47 ± 3
Aspergillus flavus (AFJ-5)	39 ± 2
Botryosphaeria obtusa (BOJ-16)	-
Botrytis cinerea (BCJ-3)	37 ± 2
Colletotrichum acutatum (CAJ-4)	51 ± 3
Colletotrichum gloeosporioides (CGJ-7)	39 ± 3
Fusarium avenaceum (FAJ-1)	15 ± 2
Monilinia fructigena (MFJ-2)	33 ± 2
Mucor piriformis (MPJ-7)	13 ± 1
Penicillium expansum (PEJ-5)	41 ± 2

[a] Growth inhibition expressed as the diameter of the inhibition zone in mm. Values are average of three independent experiments ± SD.

Table 3 Inhibition of apple fruit necrosis induced by fungal pathogens using 200 µg ml^{-1} DDHR

	Fruit necrosis (Ø mm)		
	A. alternata	*C. acutatum*	*P. expansum*
Solvent (DMSO)	-	-	-
Pathogen w/o DDHR	41.0±1.0[#]	37.0±1.0	84.5±0.5
Pathogen + DDHR	12.5±0.5*	13.5±0.5*	23.0±1.0*
% IN[##]	69.5±0.5	63.5±1.0	72.8±1.0

[#]values are average of three independent experiments±SD

[##] % IN is % of necrosis inhibition calculated according to formula: KR-R/KR x 100. KR-radius of necrosis in infected fruit (pathogen w/o DDHR), R-radius of necrosis in infected fruit and treated with 200 µg ml^{-1}DDHR (pathogen + DDHR). *Necrosis is statistically significantly inhibited with DDHR ($p<0.0001$) compared to infected fruits (pathogen w/o DDHR).

Figures

Fig. 1 Chemical structure of 32, 33- didehydroroflamycoin (DDHR;$C_{40}H_{64}O_{12}$)

Fig. 2 *In vitro* effect of DDHR on *Colletotrichum acutatum*

C. acutatum grown on PDA plate containing DMSO (A) and in the presence of 200 µg DDHR per disc (B). DDHR induced mycelia necrotic ring can be seen in panel B. Brightfield microscopy of *C. acutatum* mycelia morphology in the absence (C) and in the presence of DDHR (D). DDHR inhibits spore formation (spores are indicated with thick arrows in C but absent in D) and causes abnormal shape and necrosis of hyphae as indicated by thin arrows

(D). Pictures are representative of three independent experiments. Scale bar represents 10 µm.

Fig. 3 *In vivo* effect of DDHR on fungal infection
Apple fruits inoculated with *A. alternata* (A and B), *C. acutatum* (C and D) and *P. expansum* (E and F) conidial suspension and DDHR; (C+) positive control, fruit inoculated with fungal conidial suspension and DMSO; (T) fruit inoculated with DDHR and fungal conidial suspension; (C-) negative control, fruit with DMSO only. Pictures are representative of three independent experiments.

Fig. 4 Effect of DDHR on photosynthesis and the plant growth
Pepper chlorophyll content (SPAD units) (white bars-DMSO treatment; black bars-DDHR treated plants) (A). Root/shoot ratio (white bars-before treatment; black bars- 7 days after spraying) with DMSO (control) or DDHR (B). Values are average of three independent experiments ± SD. *Chlorophyll content and root/shoot ratio changes in DDHR-sprayed plants were not statistically significant compared to control plants (DMSO sprayed) ($p>0.05$, t-test).

Supporting Information
Fig. S1 The inhibitory effects of DHHR on mycelial growth
The agar disc of *Alternaria alternata* (A), *Colletotrichum acutatum* (B) and *Penicillium expansum* (C) was transferred to the surface of PDA plates containing different concentrations of DDHR and incubated 7 days at 25 °C. Plates containing DMSO and inoculated with test pathogens served as controls.