

ORIGINAL ARTICLE

Didehydroroflomycoin pentaene macrolide family from *Streptomyces durmitorensis* MS405^T: production optimization and antimicrobial activityN. Stankovic¹, L. Senerovic¹, Z. Bojic-Trbojevic², I. Vuckovic³, L. Vicovac², B. Vasiljevic¹ and J. Nikodinovic-Runic¹¹ Institute of Molecular Genetics and Genetic Engineering, University of Belgrade, Belgrade, Serbia² Institute for Application of Nuclear Energy (INEP), University of Belgrade, Belgrade, Serbia³ Faculty of Chemistry, University of Belgrade, Belgrade, Serbia**Keywords**

actinomycetes, antimicrobials, cytotoxicity, fungi, optimization.

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Abstract**Aims:** The aim of this study was to improve production of pentaene 32,33-didehydroroflomycoin (DDHR) in *Streptomyces durmitorensis* MS405 strain to obtain quantities sufficient for in depth analysis of antimicrobial properties.**Methods and Results:** Through classical medium optimization conditions for stable growth, DDHR production within 7 days of incubation was established. Yields of 215 mg l⁻¹ were achieved in shake flask experiments in complex medium with mannitol as the primary carbon source. DDHR had poor antibacterial activity with minimal inhibitory concentrations (MIC) of 400 µg ml⁻¹ for *Staphylococcus aureus* and *Bacillus subtilis*, while MIC of 70 µg ml⁻¹ was determined for *Candida albicans*. Using flow cytometry and fluorescent microscopy, it was demonstrated that DDHR induced membrane damage in *C. albicans* followed by cell death. Combination studies with known antifungal nystatin showed that DDHR is a promising agent for the development of novel antimycotic treatments potentially less toxic for human cells.**Conclusions:** Pentaene didehydroroflomycoin has no antibacterial activity but can be further developed for the application in antifungal therapy.**Significance and Impact of the Study:** This study is the first report on the stable and production in high yields of a novel pentaene family that acts on *Candida* cell membranes and can be used in combination with known antifungals. Polyenes are still antifungal antibiotics of choice, and therefore, isolation and production of new lead structures are highly significant.**Introduction**

Polyene macrolides are a group of bioactive molecules with large macrolactone rings containing a series of 3–8 conjugated double bonds (Omura and Tanaka 1984). They are synthesized via repetitive enzymatic condensation of small carboxylic acids by the multifunctional enzymes polyketide synthases usually as secondary metabolites of *Streptomyces* strains (Aparicio *et al.* 2003; Fischbach and Walsh 2006). Polyenes were the first agents used in antifungal therapy (Juan and McDaniel 1977) and were shown to have a broad spectrum of antifungal

activity with low frequency of resistant pathogen appearance (Ghannoum and Rice 1999). To date, more than 200 polyene macrolides have been discovered and traditionally have been considered as potential antifungal agents (Zotchev 2003). Only a few antifungals are currently being used in human therapy including amphotericin B, nystatin, candicidin, pimarinic, methyl partricin and trichomycin (Zotchev 2003). Polyene macrolides interact with sterols in the cell membrane usually forming barrel shaped structured pores making it permeable to ions and other small molecules (Bolard 1986; Milhaud 1992; Cohen 2010). Low water solubility and interactions

with cholesterol in mammalian cell membranes are accountable for their poor tissue distribution, severe side effects and high toxicity (Rimaroli *et al.* 2002; Recamier *et al.* 2010; Wasko *et al.* 2012). Despite all drawbacks, polyene macrolides make up one of the most important classes of systemic antifungals and efforts are continuously made towards obtaining new, safer and improved broad-spectrum antifungal polyene agents (Georgopapadakou 1998; Zotchev 2003; Ostrosky-Zeichner *et al.* 2010).

Due to their complex chemical structure, polyene antibiotics that are in current clinical use are produced by microbial fermentations (Jonsbu 2001; Lemke *et al.* 2005). For amphotericin B and nystatin patents for production in biotechnological processes issued in 1950s with some improvement are still the main source points for the production on an industrial scale (Hazen and Brown 1957; Dutcher *et al.* 1959; Schaffner and Kientzler 2000). Recently, a novel polyketide pentaene macrolide family with the predominant member 32,32-didehydrorflamycoin (DDHR; Fig. 1a), produced by *Streptomyces durmitorensis* MS405^T, has been described (Stodulkova *et al.* 2011). Its cell toxicity was established on the various human and mouse carcinoma cell lines (Stodulkova *et al.* 2011). However, due to the poor and unstable production and limited amounts of the metabolite available, DDHR potential as an antibacterial and antifungal agent has not been examined. Thus, we were set to optimize production conditions for DDHR and to evaluate its antibacterial and antifungal activity, as well as to establish its haemolytic activity and cytotoxicity on noncancer cell lines.

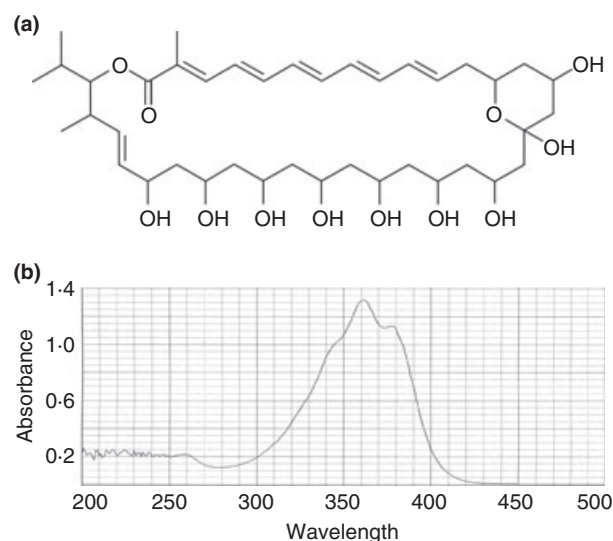


Figure 1 32,33-Didehydrorflamycoin (DDHR) chemical structure (a) and characteristic polyene UV-Vis spectrum of the ethyl acetate extract of the *Streptomyces durmitorensis* MS405 submerged culture (b).

Materials and methods

Streptomyces durmitorensis MS405 maintenance and culture conditions for DDHR production

Streptomyces durmitorensis MS405 strain (DSM 41863) (Savic *et al.* 2007) was maintained on solid mannitol soy flour medium (MSF) (Kieser *et al.* 2000). Plates were incubated at 30°C for 7 days, and spore suspension was made. *Streptomyces durmitorensis* MS405 spore suspensions were stored in glycerol (20%, v/v), maintained at –80°C and used for the inoculation of cultures for further experiments (Kieser *et al.* 2000). Spore suspensions (20 µl) were firstly inoculated into tryptone soy broth (TSB) (25 ml; TSB powder, 30 g l⁻¹) and incubated at 30°C for 48 h, and this preculture was washed with PBS and then used for the inoculation of different media (1%, v/v). For the DDHR production, cultures were grown in Erlenmeyer flasks (1 : 5 culture to volume ratio) containing coiled stainless steel spring for better aeration and unless otherwise stated incubated in dark at 30°C on a rotary shaker (200 rev min⁻¹) for 7 days. Unless otherwise stated, all media components were purchased from Oxoid (Cambridge, UK), Becton Dickinson (Sparks, MD, USA) or Sigma-Aldrich (Munich, Germany).

Composition and preparation of the media for optimization of the DDHR production

Five different complex media were tested for the growth and accumulation of DDHR by *S. durmitorensis* MS405. Complex media included in this study were as follows: NEM (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); JS (glucose, 20 g l⁻¹; soluble starch, 20 g l⁻¹ (Merck, Darmstadt, Germany); mannitol, 15 g l⁻¹; soy flour, 30 g l⁻¹ (Florida Bel, Zemun, Serbia); CaCO₃, 10 g l⁻¹; MSF (soy flour, 20 g l⁻¹; mannitol, 20 g l⁻¹; in tap water; GYM (glucose, 4 g l⁻¹; yeast extract, 4 g l⁻¹; malt extract, 10 g l⁻¹); YED (glucose, 50 g l⁻¹; Bacto-peptone, 10 g l⁻¹; CaCO₃, 10 g l⁻¹; MnCl₂, 0.01 g l⁻¹; FeSO₄, 0.01 g l⁻¹).

NEM medium was optimized by subtraction of various media components such as sugars and nitrogen sources and combinations thereof, supplementation of methyl oleate (0.2%, v/v), grape seed oil (0.2%, v/v), substitution of carbon sources with equimolar carbon amount of glucose (29.8 g l⁻¹), mannitol (30.1 g l⁻¹), glycerol (24.1 ml l⁻¹), arginine (28.8 g l⁻¹), sodium succinate (67 g l⁻¹), methyl oleate (18 ml l⁻¹) and substitution of nitrogen sources with sodium nitrate (3 g l⁻¹) (Table 2). The pH of the media was adjusted to 7.2 before sterilization. Media were sterilized at 121°C for 15 min.

Monitoring of DDHR production, purification and quantification

To monitor DDHR production, 3 ml of culture aliquots was taken at different time points and extracted with 2 ml of ethyl acetate. To isolate DDHR, 50 ml cultures was extracted using ethyl acetate (2 × 50 ml) by vigorous mixing at room temperature (1 h). Ethyl acetate extract was separated from aqueous phase and the cell debris by centrifugation (5000 g for 3 min at 4°C; Eppendorf 5804R bench top centrifuge). The wave length scan of the extract was carried out from 200 to 700 nm using UV/Visible spectrophotometer Ultrospec 3300pro (Biochrom, Cambridge, UK). To purify DDHR, the DDHR containing ethyl acetate fraction was then dried with Na₂SO₄, followed by drying under vacuo. This crude culture extract was further purified by flash chromatography. Flash chromatography employed silica gel 60 (230–400 mesh), while collected fractions were analysed by thin-layer chromatography using alumina plates with 0.25 mm silica layer (Kieselgel 60 F₂₅₄; Merck; ethyl acetate: MeOH (8 : 2) solvent system for development) and by UV-Vis spectral analysis. The following solvent system was used for the fractionation of 120–200 mg of ethyl acetate extract: ethyl acetate (150 ml), ethyl acetate and methanol (8 : 2 ratio, 150 ml), followed by ethyl acetate and methanol (1 : 1 ratio, 60 ml). Appropriate fractions were combined, dried under vacuo and weighed. To determine relative distribution of DDHR between mycelia and culture broth, ethyl acetate extraction was carried out on the cell pellet and the supernatant separately.

The purified DDHR was resolved and identified by liquid chromatography coupled by mass spectroscopy (LC-MS) analysis. The HPLC analysis was performed on a Agilent 1200 Series (Agilent Technologies, Santa Clara, CA, USA) with a Zorbax Extend C18 column (RRHT, 150 × 4.6 mm i.d.; 1.8 µm) and a diode-array detector (DAD), coupled with a 6210 time-of-flight LC/MS system (Agilent Technologies). The column temperature was 40°C with a constant flow rate of 0.5 ml min⁻¹. The mobile phase was a gradient prepared from 0.2% formic acid in water (A) and acetonitrile (B), according to the following programme: 0–0.24 min, 5% B; 0.24–10 min 5–95% B; 10–15 min 95% B; 15–15.5 min 95–5% B; 15–18.5 min 5% B. High resolution ESI MS spectra were recorded in the range *m/z* 100–2500 in positive and negative ion mode, with 4000V ion source potential and 140V of fragmentor potential.

Cell dry weight determination

Culture samples of 3 ml were taken in triplicate from the culture upon inoculation and every 24 h during 7 days

cultivation period. Culture samples were centrifuged at 5000 g for 5 min at ambient temperature (Eppendorf 5804R bench top centrifuge), dried for 24 h at 65°C and weighted on analytical scale (Sartorius, Göttingen, Germany).

Antimicrobial assays

Test organisms for the antibacterial assays were obtained from the American Type Culture Collection (ATCC) and National Collection of Type Cultures (NCTC). They included: *Bacillus subtilis* (ATCC 6633), *Enterococcus faecalis* (ATCC 29212), *Escherichia coli* (NCTC 9001), *Klebsiella pneumoniae* (ATCC 13883), *Listeria monocytogenes* (NCTC 11994), *Micrococcus luteus* (ATCC 379), *Pseudomonas aeruginosa* (ATCC 27853), *Salmonella typhimurium* (NCTC 12023), *Staphylococcus aureus* (ATCC 25923) and *Candida albicans* (ATCC 10231).

Standard disc diffusion assay was carried out for the preliminary screen using DDHR in dimethyl sulfoxide (DMSO). Briefly, late stationary phase cells of test microorganisms were diluted to OD₆₀₀ = 0.1 and spread on agar plates (250 µl), LB (Sambrook *et al.* 1989) for bacteria and Sabouraud dextrose agar (glucose 40 g l⁻¹; peptone, 10 g l⁻¹; agar, 15 g l⁻¹; pH 5.6) for *Candida*. Sterile paper discs (HiMedia Laboratories, Mumbai, India) were applied to the plate surface. Several concentrations of DDHR in DMSO were applied per disc (10 µg, 50 µg, 100 µg, 500 µg and 1 mg), and the same volume of the solvent was used as a control. As a positive control, nystatin powder (Hemofarm, Vrsac, Serbia) in amounts of 5 µg, 10 µg and 50 µg in DMSO was applied per disc. Plates were incubated at 30°C for 24 h, and zones of inhibition were measured.

The minimum inhibitory concentration (MIC) of DDHR was studied using a referent method for testing antimicrobial agents (EUCAST 2003) in 96-well microtiter plate assay. The assay allowed bacterial growth at 30°C and its inhibition to be assessed over time. A dilution series of DDHR were prepared in DMSO. Controls containing solvent were carried out in each assay. MIC was defined as the lowest concentration of compound at which no evidence of growth was observed.

Fluorescence microscopy and flow cytometry

Candida albicans cell suspension (OD₆₀₀ = 1) was treated with DDHR (25, 50 or 100 µg ml⁻¹), nystatin (5, 10 or 50 µg ml⁻¹) or combination of the two drugs (DDHR 25 µg ml⁻¹ and nystatin 5 µg ml⁻¹) for 2 h at 30°C. Following treatment, cells were washed with PBS and stained with propidium iodide (PI, 100 µg ml⁻¹; Sigma) in the dark for 30 min at 4°C. The cells were subsequently

counter stained with 1 $\mu\text{g ml}^{-1}$ 4',6-diamidino-2-phenylindole (DAPI; Sigma). After washing with PBS, 15 μl aliquots of cell suspension was transferred to a microscope slides, observed under Olympus BX51 fluorescent microscope and analysed with Cytovision 3.1 software (Applied Imaging Corp., San Jose, CA, USA). Aliquots of PI stained cells were washed twice with PBS, and the number of cells with damaged membranes was determined using flow cytometry on FACS Calibur (BD Biosciences, Oxford, UK), and the results were analysed using CellQuestPro software (BD Biosciences).

Haemolysis assay

Sheep red blood cells in PBS (1% v/v; Torlak, Belgrade, Serbia) were treated for 1 h with 1, 10 or 50 $\mu\text{g ml}^{-1}$ of DDHR at 37°C. Haemoglobin absorbance was measured at 405 nm (plate reader Labsystem Multiscan RC; MTX Lab-systems Inc., Vienna, WY, USA). The haemolysis percentage was calculated using the following equation: haemolysis (%) = 100[(Abs_{405 nm} (treated) – Abs_{405 nm} (nontreated))/(Abs_{405 nm} (0.1% Triton X-100 lysed) – Abs_{405 nm} (nontreated))].

Cytotoxicity assay

MRC5 cell line (human lung fibroblast, obtained from ATCC) was grown in humidified atmosphere of 95% air and 5% CO₂ at 37°C and maintained as monolayer cultures in RPMI-1640 supplemented with 100 $\mu\text{g ml}^{-1}$ streptomycin, 100 U ml⁻¹ penicillin and 10% (v/v) foetal bovine serum (FBS) (all from Sigma, Munich, Germany). MRC5 cells were treated with increasing concentrations (6.25, 12.5, 25, 50 or 100 $\mu\text{g ml}^{-1}$) of DDHR or nystatin for 48 h, and cytotoxicity was determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay (Hansen *et al.* 1989). Upon treatment, MTT (Sigma) (0.5 mg ml⁻¹) was added to each well and incubated for 1 h and then the supernatants were discarded. To dissolve, the dye precipitate DMSO (50 μl) was added to each well and oscillated for 15 s. Absorbance was measured at 490 nm on plate reader (MTX LabSystems Inc.). The MTT viability assay was performed two times in five replicates.

Results

DDHR production by *Streptomyces durmitorensis* MS405 in submerged cultures

All five complex media supported *S. durmitorensis* MS405 growth in submerged cultures to different levels. The highest biomass yield was achieved in the JS medium and

was from 2.2- to 7.3-fold higher in comparison with other media used (Table 1). On the other hand, characteristic 'three fingers'-like profile of polyene UV-Vis absorption spectrum was observed in ethyl acetate extracts of culture grown in three media, namely JS, MSF and NEM (Fig. 1b). The absorption spectra were in good correlation with previously reported maxima at 260 nm, 363 nm and a shoulder at 375 nm (Stodulkova *et al.* 2011). Upon purification using flash chromatography, polyene-containing fraction was analysed by liquid chromatography followed by tandem mass spectroscopy (LC-MS), and it was confirmed that major product of the polyene family was DDHR (Fig. 1a; Fig. S1). Indeed, the highest overall yields of DDHR polyene were obtained in NEM medium and were 1.8- and 1.9-fold higher in comparison with MSF and JS cultures, respectively, while no polyene production was detected in GYM and YED media under conditions tested (Table 1). DDHR was distributed between mycelia and culture broth in 3 to 1 ratio (data not shown); thus, the productivity of DDHR per mg of cell dry weight was 3.3- and 7.7-fold higher in NEM medium in comparison with MSF and JS, respectively (Table 1), and this medium was selected for further production optimizations.

Considering the high variation in DDHR production among five media, we have firstly examined the effect of carbon source on biomass and DDHR production (Table 2). As total carbon content in NEM medium was 12 g l⁻¹ (4 g from glucose and 8 g from mannitol), we have kept the carbon content at the same level and varied six different C-sources including glycerol, sodium succinate, methyl oleate and arginine (Table 2). All six C-sources supported *S. durmitorensis* MS405 growth, while DDHR was not detected in cultures containing sodium succinate and arginine as primary C-source. The highest levels of biomass achieved were when methyl oleate was used as primary C-source and was 1.2- to 2.7-fold higher in comparison with all other C-sources tested (Table 2).

Table 1 Biomass and 32,33-didehydroroflomycoin (DDHR) accumulation by *Streptomyces durmitorensis* MS405 grown in different complex media at 30°C for 7 days

Medium*	CDW, g l ⁻¹	DDHR, mg l ⁻¹	Productivity, mg DDHR mg CDW ⁻¹
JS	20.3 ± 0.7	108.6 ± 0.8	5.3
MSF	9.3 ± 0.4	114.3 ± 0.5	12.3
NEM	5.2 ± 0.3	211.4 ± 0.2	40.6
YED	3.7 ± 0.6	nd	–
GYM	2.8 ± 0.5	nd	–

CDW, cell dry weight; nd, not detected.

*Detailed composition of media is given in Materials and Methods section.

Table 2 The effect of carbon source on 32,33-didehydroroflomycoin (DDHR) production

Carbon source	CDW, g l ⁻¹	DDHR, mg l ⁻¹	Productivity, mg CDW g ⁻¹
Glucose	2.7	22.9	8.5
Glycerol	5.4	60.1	11.1
Methyl oleate	7.4	201.4	27.2
Mannitol	6.3	215.7	34.2
Sodium succinate	5.2	nd	–
Arginine	4.5	nd	–

CDW, cell dry weight; nd, not detected.

Results are average of two different experiments.

Glucose as the primary C-source supported the lowest biomass yields and the lowest DDHR production that was 9.4-fold lower in comparison with the culture when mannitol was the primary C-source (Table 2). However, mannitol as the primary C-source supported good growth, the highest DDHR overall yield and the production rates that were from 1.3- to 4-fold higher in comparison with all other C-sources (Table 2). Surprisingly, the methyl oleate as the primary C-source supported comparable biomass and DDHR overall yields to mannitol.

The effect of various NEM medium components on DDHR production was further examined by subtraction of different components and by supplementation with methyl oleate and grape seed oil (Table 3). Generally, subtraction of different media components resulted in decreased biomass and DDHR production. The highest biomass decrease of 1.8-fold in comparison with original NEM medium was observed when mannitol was not included in the medium. The DDHR was not detected in cultures when mannitol and yeast extracts were omitted, while decreased levels of 1.7- to 2.1-fold of DDHR were obtained when glucose, meat extract and casamino acids were not included in the medium (Table 3). Addition of methyl oleate and grape seed oil as supplements to the NEM medium resulted in 2.3- and 1.7-fold higher biomass yields respectively, while DDHR overall yields were not or only slightly changed in comparison with original NEM medium, resulting in 2.4- and 1.5-fold decrease in DDHR productivity per mg of cell dry weight (Table 3).

Antibacterial activity of DDHR

Purified DDHR had antibacterial properties against *Staph. aureus*, *B. subtilis*, *Ent. faecalis*, *M. luteus* and *Klebsiella pneumoniae*, with zones of growth inhibition of 20, 14, 13, 9 and 8 mm in diameter, respectively, when 1 mg of DDHR was applied to the discs. No zones of growth inhibition occurred when *E. coli*, *L. monocytogenes*, *Ps. aeruginosa* and *Salm. typhimurium* were used as

Table 3 Optimization of 32,33-didehydroroflomycoin (DDHR) production in NEM medium

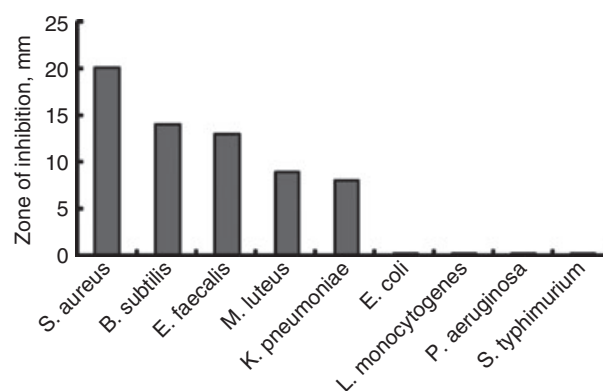
Optimization	CDW, g l ⁻¹	DDHR, mg l ⁻¹	Productivity, mg CDW g ⁻¹
Subtraction of*			
Mannitol	2.9	nd†	–
Glucose	3.8	121.4	31.9
YE	3.1	nd	–
ME	4.1	105.7	25.8
CAA	3.4	101.4	29.8
Supplementation with†			
Methyl oleate (0.2%, v/v)	11.9	200.2	16.8
Grape seed oil (0.2%, v/v)	8.6	235.7	27.4

CDW, cell dry weight; YE, yeast extract; ME, meat extract; CAA, casamino acids.

The values are mean from two experiments.

*Changes were made to NEM medium which composition is given in Materials and Methods section.

†nd, not detected.

**Figure 2** Antibacterial properties of 32,33-didehydroroflomycoin (DDHR) exhibited as zones of growth inhibition in disc diffusion assay against the panel of micro-organisms.

test organisms (Fig. 2). All tested strains were sensitive to the known antibiotic kanamycin, and in all cases, DMSO was used as a vehicle solvent and as a negative control.

The MICs, defined as minimal concentrations at which no growth occurred in liquid culture, were determined for the test organisms that showed zones of growth inhibition in disc diffusion screen (Fig. 2). The antibacterial activity of DDHR was poor, with MICs of 400 µg ml⁻¹ for *Staph. aureus* and *B. subtilis* and MICs >1 mg ml⁻¹ for other tested bacterial strains.

DDHR activity against *Candida albicans* and effect on cellular membrane

The disc diffusion assay on *C. albicans* with 100 µg of DDHR applied per disc gave 15 mm zones of growth

inhibition, while MIC determined in liquid culture was $70 \mu\text{g ml}^{-1}$. Nystatin which had a MIC of $10 \mu\text{g ml}^{-1}$ was used as a control.

As macrolide polyenes are well known to bind and disturb membranes, we studied the effect of purified DDHR on the membrane integrity of sheep red blood cells. We monitored the release of haemoglobin from RBCs treated with increasing concentrations of DDHR and showed that DDHR induces dose-dependent haemolysis with 75% cells lysed at DDHR concentration of $50 \mu\text{g ml}^{-1}$ (Fig. 3a). Next, we examined the effect of DDHR on *C. albicans* membranes by the combined PI/DAPI cell staining assay. PI is membrane impermeable dye and binds to nucleic acid only in the dead cells yielding fluorescence in the red wavelength region. DAPI easily passes the membrane and strongly binds to DNA of both living and dead cells. We treated *C. albicans* with DDHR doses below ($50 \mu\text{g ml}^{-1}$) and above ($100 \mu\text{g ml}^{-1}$) MIC concentration and showed that treatment with doses higher than MIC induced the membrane damage and cell death (Fig. 3b). *Candida* cells treated with 5 or $10 \mu\text{g ml}^{-1}$ of

nystatin had intact membranes although the cell death was visible according to brightly stained condensed nuclei indicating apoptosis (Fig. 3c).

The number of PI positive *Candida* cells treated with DDHR or nystatin was determined by flow cytometry. DDHR treatment above MIC concentration ($100 \mu\text{g ml}^{-1}$) induced the membrane damage in 80% of treated cells, while nystatin treatment resulted in only 10% cells with disintegrated membranes (Fig. 3c). These results suggested that toxic activity of DDHR differs from nystatin which is known to induce cell death by forming transient pores in the membranes of *C. albicans* (Bolard 1986; Recamier et al. 2010). Therefore, we examined the effect of two polyenes on *Candida* cells in combination (Fig. 4). Concentrations of $25 \mu\text{g}$ per disc of DDHR or $5 \mu\text{g}$ per disc of nystatin preparation separately showed neither growth inhibition zone nor membrane damage and cytotoxicity on *C. albicans* (Fig. 4a,b). However, when applied in combination they caused the growth inhibition and the membrane disintegration as demonstrated by PI positive cells with condensed nuclei (Fig. 4c).

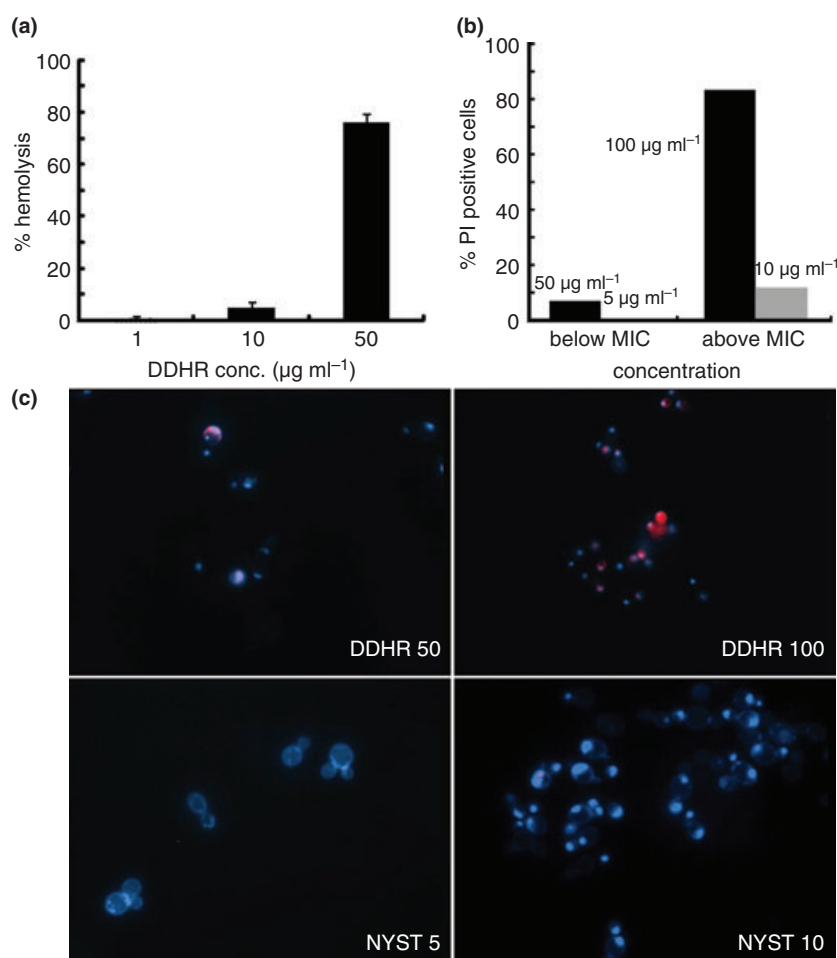


Figure 3 32,33-Didehydroroflamycoïn (DDHR) causes membrane damage. (a) One per cent sheep RBCs were incubated with DDHR, and haemolysis was followed by the increase in haemoglobin absorbance at 540 nm and quantified as percentage of Triton X-100 treated per total lysed cells. (b) Fluorescent microscopy of *Candida albicans* treated for 2 h with DDHR or nystatin and stained with 4',6-diamidino-2-phenylindole (DAPI) (blue) and propidium iodide (PI) (red). (c) FACS analysis of PI stained *C. albicans* treated with DDHR [■; 5 or $100 \mu\text{g ml}^{-1}$, concentrations below or above minimal inhibitory concentration (MIC)] or nystatin [■; 5 or $10 \mu\text{g ml}^{-1}$, concentrations below or above MIC) for 2 h.

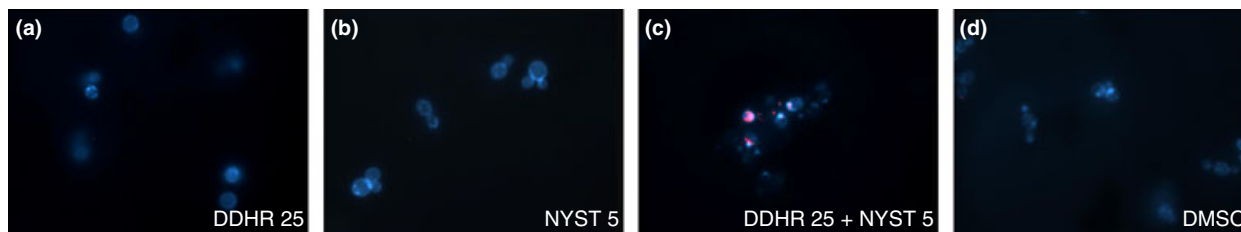


Figure 4 Fluorescent microscopy of *Candida albicans* cells treated with 25 $\mu\text{g ml}^{-1}$ 32,33-didehydroroflamycoin (DDHR) (a), 5 $\mu\text{g ml}^{-1}$ nystatin (b) or combination of the two compounds – 25 $\mu\text{g ml}^{-1}$ DDHR and 5 $\mu\text{g ml}^{-1}$ nystatin (c) and stained with 4',6-diamidino-2-phenylindole (DAPI) (blue) and propidium iodide (red). Dimethyl sulfoxide treated cells were presented as a negative control (d).

Cytotoxicity of DDHR to human cells

To address the suitability of DDHR for the treatments of candidemia in humans we finally examined its cytotoxicity to human cells. DDHR killed HTR-8/SVneo human trophoblast cell line (Graham *et al.* 1993), although less efficiently with the IC_{50} of 1 mmol l^{-1} determined after 1 h of treatment (data not shown). We treated fibroblast cell line MRC5 with increasing concentrations of DDHR and observed cell viability after 48 h of treatment using MTT assay. DDHR induced fibroblast death only when the doses above *Candida* MIC concentration were applied (Fig. 5). *Candida* MIC concentrations for nystatin nystatin (>10 mg ml^{-1}) did not have any toxic effect on MRC5 cells.

Discussion

From early on, since their discovery, it was noticed that production of polyene antibiotics in *Streptomyces* greatly depended on media composition and cultivation conditions (Martin and McDaniel 1977; Jonsbu *et al.* 2000, 2002; Martin *et al.* 2011). Indeed, from our experience with *S. durmitorens* MS405, it took a considerable period of time after the strain isolation and bioactive compound exhibiting activity on *Saccharomyces cerevisiae* FAV20 strain was detected on solid media (Savic *et al.* 2007) that the structure of the bioactive compound was elucidated and limited activity shown (Stodulkova *et al.* 2011). This was due mainly to unsteady growth and production of the compound in submerged culture. Therefore, the first objective of this study was to establish submerged culture conditions to allow high and steady pentaene macrolide DDHR family production in this strain to obtain sufficient material for further activity tests.

Streptomyces durmitorens MS405 grew poorly in minimal media and in liquid media widely used as standard for propagation of soil actinomycetes such as R2, YEME or TSB (Kieser *et al.* 2000). It usually took 28 days of incubation in TSB medium supplemented with mannitol for the production of DDHR (Stodulkova *et al.* 2011).

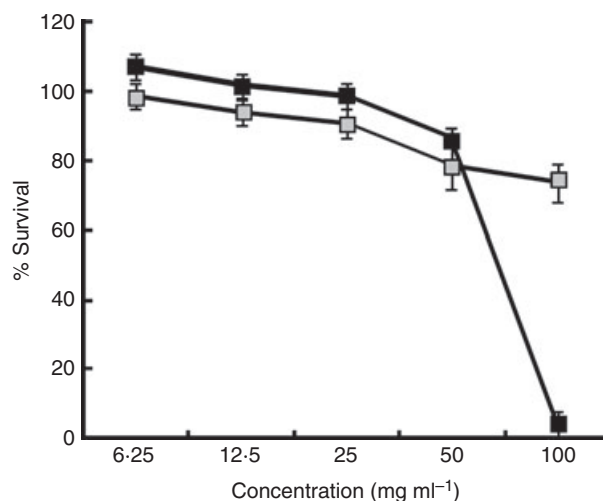


Figure 5 Cytotoxicity (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay on human fibroblasts treated for 48 h with 32,33-didehydroroflamycoin (DDHR) (■) or nystatin (▒).

In this study, growth and DDHR production were tested in five complex nutrient-rich media (Table 1). In the JS or MSF medium, containing complex nutrients as soy flour, soluble starch and mannitol, high biomass and production of DDHR was achieved even after 5 days incubation, while in more defined media such as GYM or YED containing glucose and yeast extract or peptone, biomass yields were 5–7 folds lower with no polyene production (Table 1). However, the medium that stood out for the highest reproducibility of the both biomass and DDHR yields was between the two groups, containing yeast extract, beef extract, casamino acids, glucose and mannitol (NEM). The synthesis of DDHR was highly dependent on the presence of mannitol in the medium.

The amount and type of carbon source dependency for growth and bioactive compounds production in *Streptomyces* is not unusual and appears that special nutrition requirements are the rule rather than the exception in polyene production by *Streptomyces* (Liu *et al.* 1975;

Jonsbu *et al.* 2000, 2002). Of all tested carbon sources, mannitol and methyl oleate gave equally high production of DDHR, whereas use of glycerol and glucose gave significantly lower yields of 3.4- and 9-folds, respectively. Substances with long aliphatic chain such as methyl oleate had previously been used as the supplement for the improvement of other polyketide yields (Frykman *et al.* 2005), and other pentaenes, namely filipin III (Brock 1956).

Overall yields of DDHR were 50-fold higher in comparison with similar shake flask cultivations of *Streptomyces filipinensis* for filipin III production (Brock 1956) or *Streptomyces nodosus* ATCC14899 for amphotericin B production (Nikodinovic 2004). Yields obtained in this study were about 3-fold higher in comparison with yields of tetraene nystatin obtained in batch fermentation (Jonsbu *et al.* 2001, 2002) and about 2-fold higher in comparison with amounts of heptaene trichomycin B obtained from the fermentor cultivation (Komori 1990). However, DDHR yields were 15- to 20-folds lower in comparison with industrial scale production yields reported for heptaenes amphotericin B and candicidin, respectively (Dutcher *et al.* 1959; Liu *et al.* 1975; Gil *et al.* 1985; McNamara *et al.* 1998). Further increase in DDHR production could be achieved by genetic engineering or manipulation of the availability of precursors for synthesis of secondary metabolites (Olano *et al.* 2008; Martin and Liras 2010; Martin *et al.* 2011).

Although polyenes are known as the first choice in antifungal treatment, their amphipathic structure allows functional versatility that cannot always be predicted – polyene macrolides such as nystatin and amphotericin B do not show any considerable antibacterial activity, but structurally related faeriefungin is active against gram positive, and some gram negative strains (Mulks *et al.* 1990). Once we had established conditions for efficient production and isolation of DDHR, it allowed further detailed examination of its antibacterial properties. With MICs higher than 400 $\mu\text{g ml}^{-1}$ against bacterial strains, it was concluded that DDHR had no significant antibacterial effect. However, MICs of 70 $\mu\text{g ml}^{-1}$ against *C. albicans* prompted further mechanistic studies. MICs of other polyene antifungals against a range of *C. albicans* strains and clinical isolates vary greatly from 0.03 $\mu\text{g ml}^{-1}$ for trichomycin (Komori 1990), 50 $\mu\text{g ml}^{-1}$ for filipin (Hamilton-Miller 1973), 0.5 $\mu\text{g ml}^{-1}$ to 1 mg ml^{-1} for amphotericin B (EUCAST 2010), 0.54 $\mu\text{g ml}^{-1}$ (Carrillo-Munoz *et al.* 1999; Arikan *et al.* 2002) and 5.29 $\mu\text{g ml}^{-1}$ for nystatin (Sousa *et al.* 1985).

To investigate the mechanism of DDHR-induced cytotoxicity, we firstly examined the membrane integrity of DDHR-treated RBCs. Our results demonstrated that unlike natamycin which interferes with endocytosis but

does not permeabilize (Van Leeuwen *et al.* 2009), or nystatin that induces apoptosis by forming transient pores in the membranes of treated cells, DDHR induces membrane damage because it causes dose-dependent haemolysis. In addition, we showed here that DDHR kills *Candida* cells by inducing membrane disruption because cell death was only observed in cells with damaged membranes. The effect that DDHR has on cell membranes could be similar to structurally similar filipin which forms micellar complexes with sterols that integrate into lipid bilayer changing its permeability and causing its disruption (Aparicio *et al.* 2004). However, the more detailed mechanistic studies have to be performed to elucidate the exact mechanism of how DDHR interacts with the membrane and causes damage.

Stodulkova *et al.* reported that DDHR colocalized with late endosomal/lysosomal markers in HeLa cells and induced apoptosis after 4 h of treatment with IC₅₀ (concentration of the drug causing 50% inhibition of the cell viability) varying between 60 and 100 $\mu\text{mol l}^{-1}$ (Stodulkova *et al.* 2011). The cytotoxicity assay on fibroblast cell line presented here confirmed the cytotoxic effect of DDHR at relatively high concentrations (Fig. 5). Polyenes bind membrane sterols whose presence influences the formation of pores and the effect that polyenes have on particular cell (Recamier *et al.* 2010). Polyenes have higher affinity for ergosterol which is primarily constituent of fungal membranes, while human cell membranes are richer in cholesterol (Teerlink *et al.* 1980). This could explain the different effects of DDHR observed on mammalian in comparison with fungal cells. In this sense, DDHR should be developed in the direction of antifungal drugs not an antitumor agent as has been the case for other polyenes (Vaishnav and Demain 2011). It was suggested that a possible approach in overcoming antifungal drug resistance was to combine two or three classes of antifungals preferably the ones with different mechanisms of action (Vazquez 2007). Combination of DDHR with one of the antifungal drugs with different mode of action could be a good way for improvement of conventional antimycotic therapies making them more efficient and less toxic to human cells.

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Conflict of interest

No conflict of interest declared.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1 HPLC profile of ethyl acetate extract of *Streptomyces durmitorensis* MS405 culture grown in NEM medium and (+)-HR ESI TOF MS of the most abundant compound of the extract (32,33-didehydroroflamycoin, DDHR). The adduct ions $[M+Na]^+$ at m/z 759.3460 and $[2M+Na]^+$ at m/z 1495.7045 could be observed. Also, a cluster of ions corresponding to the loss of water molecules (from two up to seven) from the molecular ion $[M+H]^+$ were present.