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ORIGINAL PAPER

Production and genetic improvement of a novel antimycotic agent, Saadamycin, against Dermatophytes and other clinical fungi from Endophytic *Streptomyces* sp. Hedaya48

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Abstract As a part of our ongoing efforts towards finding novel antimycotic agents from marine microflora of the Red Sea, vanillin, 5,7-dimethoxy-4-*p*-methoxylphenylcoumarin and the new antimycotic compound saadamycin were isolated from endophytic *Streptomyces* sp. Hedaya48. The producing strain was isolated from the Egyptian sponge *Aplysina fistularis* and subjected to different UV irradiation doses. A mutant strain Ah22 with 10.5-fold (420 mg/l as compared to 40 mg/l produced by the parental strain) improved saadamycin production was isolated. Production of saadamycin from mutant Ah22 was enhanced to 2.26-fold (950 mg/l) and 2.38-fold (1000 mg/l) under optimized culture conditions in batch culture and bioreactors, respectively. Both saadamycin and 5,7-dimethoxy-4-*p*-methoxylphenylcoumarin exhibited significant antimycotic activity against dermatophytes and other clinical fungi.

Keywords *Aplysina fistularis* · Endophytic · *Actinomycetes* · Dermatophytes · Mutation

Introduction

Dermatophytes are a group of fungi responsible for causing dermatophytoses in humans, and there is evidence that the dermatophytes have acquired resistance to certain antimycotic drugs [22]. As fungi are eukaryotic and have biosynthetic machinery similar to that of higher animal, agents that inhibit protein, RNA or DNA biosynthesis in fungi have greater potential toxicity to the host [9]. Therefore, finding new effective and safe antifungal agents that selectively inhibit clinically important fungi without exhibiting any toxicity to humans is a major challenge to the pharmaceutical industry today [11].

Streptomycetes are the source of about 80% of the antibiotics therapeutically used, i.e., aminoglycosides, cephamycins, polyenes, tetracyclines, macrolides, benzopyrones and triazolopyrimidine antibiotics [5, 7, 27]. A variety of relationships exist between endophytes and their hosts ranging from mutualistic, symbiotic or antagonistic. Because of what appears to be their contribution to the host, the endophytes may produce a plethora of substances that possess unique structures, representing a huge reservoir of bioactive compounds available to modern medicine [21].

Induction of mutation followed by selection of mutants is still an important approach in the improvement programs of all strains [4, 16]. The exposure of different species of *Streptomycetes* to mutagenic agents has led to the development of high producer strains, for example, *Streptomyces noursei* mutants for production, of nystatin and other antibacterial antibiotics [13].

2-Pyrone is a six-membered cyclic unsaturated ester, which is highly abundant in bacteria, plant, insect and animal systems and takes part in many different types of biological processes such as defense against other organisms and participation in key biosynthetic reactions as an intermediate or metabolite [20].

We report here the taxonomy of the antibiotic-producing strain and the improvement of antibiotic production by genetic tools via UV irradiation followed by optimization

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of production conditions. Isolation, structural elucidation and antimycotic activity of the saadamycin antibiotic from the mutant strain of *Streptomyces* sp. Hedaya48 are also described.

Materials and methods

General experimental procedures

UV spectra were recorded with a Beckman DU 640 spectrometer and IR spectra with a JASCO FT/IR-430 instrument. The melting points were determined on a Buchi-540 melting-point apparatus. 1D and 2D NMR spectra were recorded on a Bruker DRX-500 instrument at 500 MHz for ^{1}H NMR and 125 MHz for ^{13}C NMR. Low- and high-resolution mass spectra were recorded respectively on Shimadzu LCMS-8000QPa and JEOL HX110A spectrometers.

Sponge material

Healthy specimens of the Egyptian sponge *Aplysina fistularis* were collected from Sharm El-Sheikh from January to February 2008. Taxonomic identification of the sponge was performed by the National Institute of Oceanography and Fisheries Research Station, Hurghada, Egypt.

Isolation of the antibiotic producer strains

The endophytic *Actinomycetes* strains were separated from the inner healthy tissue of *Aplysina fistularis* sponge according to the procedure described by Taechowisan and Lumyong [25] with slight modification. The samples were washed with distilled water and cut into small pieces of ca. 4×4 mm^2. Tissue pieces were rinsed in 0.1% Tween 20 for 1 min, then in 2.5% sodium hypochlorite for 15 min followed by washing in sterile distilled water for 5 min. The surface was sterilized with 75% ethanol for 5 min then rinsed in sterile water three times. Finally the pieces were transferred to dishes of starch casein agar containing 100 μg/ml nystatin and cycloheximide to inhibit fungal growth until bacterial mycelium or colonial growth arose from the injury surface, and plates were then incubated at 30°C for 2 weeks. Single colonies were transferred periodically to the same medium, and after 7 days, pure cultures of *Actinomycetes* strains were obtained and examined for anti-mycotic substance production.

Characterization of the hyper-producer isolate

The hyper-antibiotic-producing strain Hedaya48 was identified by the analysis of phenotypic and chemotypic characteristics, and the 16S rDNA sequence. Morphological and cultural characterization was done according to the diagnostic key of Szabo et al. [24] and Williams et al. [29], and according to the International *Streptomyces* Project (ISP) Scheme as described by Shirling and Gottlieb [23].

Determination of the isomer of diaminopimelic acid (DAP) and the whole-cell sugar pattern was carried out as described by Hasegawa et al. [10]. Fatty acid methyl esters were prepared by the trimethylsulphonium hydroxide method [1]. The base composition of genomic DNA of Hedaya48 strain was determined by the method of Mandel and Marmur [18].

16S rDNA sequencing

Genomic DNA was extracted and purified using the QIAGEN DNeasys Tissue Kit following the manufacturer's protocol for gram-positive bacteria and animal tissue. Amplification of ribosomal DNA was performed using puReTaqTM Ready-To-GoTM PCR Beads (Amersham Biosciences). For amplification of the nearly complete 16S rRNA gene, the eubacterial primers 27f and 1492r were used. The conditions for this PCR were: initial denaturation (2 min at 94°C) followed by 45 cycles of primer annealing (40 s at 50°C), primer extension (90 s at 72°C) and denaturation (40 s at 94°C), a final primer annealing (1 min at 42°C) and a final extension phase (5 min at 72°C). PCR products were checked for correct length on a 1% Tris–borate-EDTA (TBE) agarose gel (1% agarose, 8.9 mM Tris, 8.9 mM borate, 0.2 mM EDTA), stained with ethidium bromide and visualized under UV illumination [26]. Sequence data were edited with Lasergene Software SeqMan (DNAStar Inc.). Next relatives were determined by comparison to 16S rRNA genes in the NCBI GenBank database using BLAST (Basic Local Alignment Search Tool).

Clinical target fungal isolates

Clinical fungal isolates of *Epidermophyton floccosum, Trichophyton rubrum, Trichophyton mentagrophytes, Microsporum gypseum, Aspergillus niger, Aspergillus fumigatus, Fusarium oxysporum, Candida albicans and Cryptococcus humicolus* were provided by Tanta University Hospitals, Egypt. Fungi were maintained on SDA (Sabouraud Dextrose Agar) and yeasts on YMA (Yeast Malt extract Agar) media to be used as target organisms for this study.

Antibiotic bioassay

This was carried out according to the paper-disc diffusion method using SDA and YMA media for fungi and yeast, respectively [12], and then incubated for 48–96 h at 37°C. The inhibition zone diameter was measured in mm, and the

antibiotic concentration (μg/ml) was determined using a standard calibration curve based on the purified antimycotic substance (saadamycin) produced by Hedaya48 strain.

Induction of mutation by UV irradiation

Spores of Hedaya48 were gently scraped from the surface of ISP-2 agar plates, washed with sterile normal saline (0.90%) and filtered through glass wool. Spore suspensions were checked microscopically and diluted to have a count of 10^4 spore/ml. Three milliliters of spore suspension was exposed to UV light (Philips TUV 30-W lamp) for different exposure times (5, 10, 15, 20, 25, 30, 35 and 40 min) placed about 25 cm above the liquid surface and gently swirled in a petri dish. After incubation in the dark, spores were plated on ISP-2 agar, incubated at 28°C and observed after 72 h. Mutation, survival rates and antibiotic production were determined.

Selection of wild and mutant strains for antibiotic production

For antibiotic production, a survey of 50 local *Streptomyces* isolates as well as 100 mutant colonies of the hyper-antibiotic producer wild strain, Hedaya48, was carried out in triplicate in 250-ml Erlenmeyer flasks containing starch-nitrate broth as a basal medium. It was composed of (g/l): starch, 10.0; $NaNO_3$, 2.0; K_2HPO_4, 1.0; KH_2PO_4, 1.0; $MgSO_4{\cdot}7H_2O$, 0.5; KCl, 0.5; seawater, 1.0 l; pH was adjusted to 7.0.

Determination of dry weight

The cells were separated from the culture filtrate by centrifugation at 5,000 rpm for 15 min, washed twice with distilled water and then dried at 60°C until reaching a constant weight.

Optimization of saadamycin production

The optimization of production of the anti-mycotic antibiotic, saadamycin, was carried out in 250-ml Erlenmeyer flasks containing 50 ml of starch nitrate medium and monitored in terms of μg/ml. Duplicate flasks were pooled for analysis, and each result was an average of triplicate assays. Each parameter optimized earlier was incorporated in subsequent experiments.

Typical time course of antibiotic production by Ah22 mutant and parent strain

Antibiotic production by the parent strain Hedaya48 and its mutant Ah22 was determined periodically during 14 days of fermentation using starch nitrate broth medium.

Effect of different cultivation media

To determine which medium stimulates maximum anti-fungal activity, 250-ml Erlenmeyer flasks containing 50 ml each of the following media separately were inoculated to 10^6 spores/ml and incubated at 28°C and 180 rpm: medium 1, potato dextrose broth (PDB); medium 2, malt yeast peptone broth (MYPB); medium 3, glycerol asparagine; medium 4, starch nitrate; medium 5, composed of (%) starch 1, yeast extract 0.4, peptone 0.2; and medium 6 containing (%) starch 1, glucose 0.3, malt extract 1, yeast extract 0.5. The antibiotic activity was extracted with EtOAc and determined periodically after 3, 6, 9, 12 and 15 days of fermentation.

Effects of different carbon and nitrogen sources on antibiotic production

The effect of the carbon source on antibiotic production was investigated by replacing the carbon source of the basal medium (Starch) by different sole carbon sources at a concentration of 2%. Different nitrogen sources and amino acids were evaluated as the sole nitrogen source for antibiotic production instead of $NaNO_3$ at a final concentration of 0.2% in medium containing a mixture of 1% glucose and 1% starch as carbon source.

Effects of different metal ions

Magnesium (Mg^{2+}), ferrous (Fe^{2+}), zinc (Zn^{2+}) and copper (Cu^{2+}) ions in the form of sulfate, manganese (Mn^{2+}), potassium (K^+) and sodium (Na^+) ions in the form of chloride, calcium (Ca^{2+}) ions in the form of carbonate and phosphate (PO_4) in the form of potassium hydrogen phosphate were tested to investigate the effect of these ions on the production yield using modified starch nitrate medium composed of (g/l): starch, 10; glucose, 10; $NaNO_3$, 1.0; valine, 0.5; alanine, 0.25; phenylalanine, 0.25; K_2HPO_4, 1.0; KH_2PO_4, 1.0; $MgSO_4{\cdot}7H_2O$, 0.5; KCl, 0.5; seawater 1 l. Each metal ion under study was added at a concentration of 0.02, 0.05, 0.1, 0.2 and 0.3% instead of its usual concentration in this medium, and incubated for 6 days.

Temperature and pH

Seven 250-ml Erlenmeyer flasks, each with 50 ml of the modified medium as mentioned above, were inoculated with Ah22 spores at a concentration of 10^6spores/ml. Flasks were incubated at different temperatures viz., 20, 25, 30, 35, 37, 40 and 45°C on a rotary shaker for 6 days.

The initial pH of the cultivation medium was adjusted to 3, 4, 5, 6, 7, 8 and 9. All flasks were inoculated as

mentioned above and incubated at 35°C on a rotary shaker (180 rpm) for 6 days.

Extraction and isolation of saadamycin

The fermented broth of the Ah22 mutant (5 l) was collected and extracted with ethyl acetate (1:1, v/v). The mixture was shaken overnight and allowed to stand for 60 min to separate the solvent from aqueous phase. The organic extract was separated, dried over anhydrous sodium sulfate and concentrated under reduced pressure to yield 7.9 g as crude fungicidal extract. The extract was partitioned between hexane and 60% aqueous MeOH, and then the fungicidal aqueous MeOH fraction was extracted with $CHCl_3$. Evaporation of $CHCl_3$ extract was done under reduced pressure to yield a pale yellowish white solid (6.12 g), which was subjected to gel chromatography on a silica gel (60–120 mesh) column eluted with a linear gradient of hexane and ethyl acetate. Fractions eluted with hexane and ethyl acetate (7:3) were found to be pure fractions, and then collected to give compound 1 (vanillin, 10 mg/l). Fungicidal fractions were combined, concentrated and further fractionated on a column of Sephadex LH-20 (40.0 g) eluted with a linear gradient of CH_2Cl_2: MeOH. Fractions eluted with 0.1 and 0.4 MeOH in CH_2Cl_2 were found to be pure fungicidal fractions. The same pure fractions were combined and evaporated to yield 5,7-dimethoxy-4-*p*-methoxylphenylcoumarin (80 mg/l) and saadamycin (750 mg/l), respectively, as major ingredients of the culture broth of the producing mutant.

Determination of minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) values of saadamycin against fungi

The MIC values of saadamycin and the reference antimycotic agent myconazole were determined by the broth tube dilution procedure using two-fold dilution in Sabouraud dextrose broth (SDB) at 37°C for 96 h; the MIC was determined as the lowest concentration of saadamycin that showed no visible growth [2]. MFC values of saadamycin and myconazole were determined by sub-culturing 50 ml from tubes not visibly turbid and spot inoculating it onto SDA plates. MFC values were determined as the lowest concentration that prevented growth on subculture [17].

Results and discussion

Isolation and screening of bioactive *Actinomycetes*

Out of 50 endophytic *Actinomycete* isolates obtained from sponge samples, 10 isolates (20%) exhibited antimycotic metabolites against the clinical fungi under study. Among the producer isolates, the strain designated Hedaya48 was a potent producer and was selected for further studies. Many authors have suggested that endophytes are rich sources of bioactive products such as enzymes, anti-microbial, anti-cancer and anti-malarial agents [8]. Moreover, Taechowisan and Lumyong [25] have reported that secondary metabolites of endophytic *Actinomycetes* of *Zingiber officinale* and *Alpinia galangal* were very active against phytopathogenic fungi.

Taxonomic classification of hyper-producer strain

The morphological and physiological characteristics of the Hedaya48 strain, as well as its cell-wall type, whole-cell sugar pattern, fatty acid profile and the sequence of its 16S rRNA gene, are consistent with the characteristics of members of the genus *Streptomyces*. Strain Hedaya48 produced a blue-red to violet-red substrate mycelium and a blue spore mass composed of spiny spores in spirals-type spore chains as revealed by scanning electron microscopy (Fig. 1).

Chemotaxonomic analysis showed that the cell wall contained D-alanine, glycine and LL-DAP, indicating that it has cell-wall type I. Mannose, galactose, glucose and ribose were detected as diagnostic sugars in the hydrolysates of whole cells. Fatty acid analysis showed that strain Hedaya48 contained a high proportion of saturated straight chain, iso- and anteiso-branched fatty acids: Iso- C14:0 (7.65%), C12:0 (6.39%), C15: 0 (23.12%), C18: 0 (8.74%), C18: 1 (10.24%), C18: 2 (22.42%) and anteiso-C16: 0 (21.44%). The G+C content of the genomic DNA was 71.2%.

Strain Hedaya48 differs from other *Streptomycetes* that produce a blue spore mass in several respects including similarity of 16S rRNA genes. Strain of *Streptomyces amakusaensis* (1,451-bp 16S rDNA sequence by pair-wise

Fig. 1 Spore surface ornamentation of *Streptomyces* sp. Hedaya48 strain

alignment of *S. amakusaensis* shows only 92% similarity to the strain Hedaya48) differs from strain Hedaya48 in producing smooth, blue spores in spiral spore chains and yellow-brown substrate mycelium. This species does not grow at 45°C and is sensitive to penicillin G. It is unable to grow in the presence of 7% NaCl, or with sucrose and L-rhamnose as sole carbon sources, or L-histidine as the sole nitrogen source [29]. *Streptomyces glaucescens* (showed 80% similarity with Hedaya48) produces a red-orange substrate mycelium and red-orange diffusible pigments as well as melanin. It is sensitive to penicillin G and cannot grow in the presence of 7% NaCl or with raffinose or D-melibiose as sole carbon sources [29]. *Streptomyces lomondensis* (showed 85% similarity with Hedaya48) produces warty to spiny blue spores in rectiflexibiles or retinaculiaperti spore chains. The substrate mycelium is brick-red, rust-brown or straw-colored with a brown or pink diffusible pigment. Melanin is produced, and casein is not degraded.

Streptomyces viridochromogenes (showed 87% similarity with Hedaya48) produces green substrate mycelium, melanin and green pH-sensitive diffusible pigments. This species is sensitive to penicillin G and cannot grow in the presence of 7% NaCl, or with sucrose as sole carbon source or L-phenylalanine as sole nitrogen source [29]. *Streptomyces tricolor and Streptomyces violaceoruber* (showed 84% similarity with Hedaya48) produce blue substrate mycelia, but differ from Strain Hedaya48 in producing smooth spores, and the spore mass is not blue [29]. These differences support the other evidence that strain Hedaya48 is not a strain of *S. amakusaensis, S. glaucescens, S. lomondensis, S. viridochromogenes, S. tricolor or S. violaceoruber.* The results support classification of strain Hedaya48 as a novel strain of *Streptomyces*. Additional data from the phenotypic characterization of the Hedaya48 strain are presented below (Table 1).

Induction of genetic variability in *Streptomyces* sp. Hedaya48

UV light induces a higher mutation frequency and a wider mutation spectrum than other mutagenesis techniques. It is the most popular method used in the induction of mutation [4, 16]. Survival rate percentages for *Streptomyces* sp. Hedaya48 decreased gradually with increasing UV exposure times (Fig. 2). In contrast, the rate of appearance of morphological variants first increased and then decreased after 25 min UV exposure times.

After the Hedaya48 strain was irradiated by UV light, mutants that differed from the original strain not only in having a different yield of antifungal activity, but also by taxonomic properties such as color of mycelium, diffusible pigments and utilization of different carbon or nitrogen sources were obtained. Out of 100 surviving colonies, 10 mutants were selected that produced the highest yield of antifungal metabolites (Table 2). Four mutants as a result of 10 min UV exposure time (Ah22, Ah25, Ah30 and Ah32) showed 10.5-, 2.4-, 3.5- and 5.1-fold increased antifungal production. Moreover, two mutants as a result of 15 min UV exposure time (Ah38 and Ah45) produced 1.9- and 4.8-fold increased antimycotic activity. Three mutants as a result of 20 min UV exposure time (Ah53, Ah60 and Ah62) produced 1.6-, 3.8- and 2.3-fold higher antifungal activity than the parent strain, respectively, and one mutant as a result of 30-min exposure time (Ah69) produced 1.2-fold more activity (Table 2). The average yield of the antifungal metabolite saadamycin was dramatically increased from 40 mg/l in wild-type to 420 mg/l in the Ah22 mutant, and the antibiotic production remained stable during 20 rounds of cultivation in shake flasks. Accordingly, Ah22 was selected for further investigation as a stable hyper-producer mutant. Wieczorek and Mordarski [28] treated *Streptomyces olivaceus* with UV light and isolated mutants that differed from the original strain in both spectrum of antimicrobial activity and taxonomic properties. Moreover, production of bioactive pyrone derivatives was enhanced in different marine *Streptomyce* species by application of genetic tools [6].

Optimization of saadamycin production

The production of the antifungal saadamycin by mutant Ah22 has been evaluated with respect to the culture conditions and the nutritional requirements that optimize production.

Time course of antifungal saadamycin production

The fermentation characteristics of the parental strain Hedaya48 and its mutant strain Ah22 in a starch-nitrate medium were monitored over a period of 14 days (Fig. 3). The fermentation time for optimal saadamycin production was shortened from 10 days for the wild strain to 6 days for mutant Ah22, which provides great advantages in industrial production by increasing efficiency. Saadamycin production by the Ah22 mutant strain was not detected during the first 24 h of fermentation; production started at day 2 (20 μg/ml) to reach its maximum value at day 6 (420 μg/ml).

Effect of different fermentation media on antifungal production by mutant Ah22

The data in Fig. 4 showed that medium 4 (starch nitrate broth) and medium 6 (SGMY) stimulated maximum production of antifungal activity by the hyper-producer mutant

Table 1 Phenotypic and chemotypic characteristics of Hedaya48 strain

Cultural characteristics on	Growth	Color of colony	Soluble pigment
ISP2	Luxurious	Deep blue	Reddish blue
ISP3	Good	Violet blue	Pale blue
ISP4	Good	Deep blue	Reddish blue
ISP5	Luxurious	Deep blue	Light blue
ISP6	Good	Deep reddish blue	Reddish blue
ISP7	Good	Deep pink blue	Light blue
Glucose-asparagine agar	Luxurious	Yellowish blue	Dark blue
Czapek's agar	Good	Deep blue	Blue red
Nutrient agar	Good	Medium blue	Pale blue
Physiological and biochemical characteristics		Utilization of (1%)	
		Adonitol	–
Melanin production	–	L-Arabinose	+
H_2S production	–	D-Galactose	+
Cell wall amino acids	D-Ala, Gly, 2, 6- DAP	D-Mannose	+
Whole cell sugars	Man, Gala, Glu, Rib.	D-melibiose	+
Major fatty acids (%)		L-Rhamnose	+
Iso- C14:0	7.65	L-Sorbose	–
C12:0	6.39	Mannitol	+
C15:0	23.12	N-acetyl glucosamine	+
Anteiso-C16:0	21.44	Lactose	+
C18:0	8.74	Sucrose	+
C18:1	10.24	Maltose	+
C18:2	22.42	Raffinose	+
Characteristic phospholipids		Malate	+
DPG	+	Citrate	–
PI	+	Oxalate	+
PE	+	Utilization of	
PIMS	+	Casein	+
Aerobic reduction of $NaNO_3$	+	L-Histidine	+
Tyrosinase activity	+	L-phenylalanine	+
Hydrolysis of		Sensitivity to antibiotics	
Starch	+	Tetracycline	+
Cellulose	–	Streptomycin	–
Casein	+	Gentamycin	–
Gelatine	+	Erythromycin	–
Utilization of (1%)		Chloramphenicol	+
Glucose	+	Penicillin G	–
Glycerol	+	Growth at 45°C	+
Erythritol	–	Optimum NaCl for growth (%)	15–25
		Mol% G+C	71.2

Ah22 (420 and 380 μg/ml, respectively) after 6 days of fermentation at 28°C and 180 rpm.

Influence of carbon source

Mutant Ah22 was able to grow in all tested carbon sources (Table 3). However, substitution of starch by a mixture of 1% glucose and 1% starch showed a 21.9% increase of antifungal production. Cultures containing galactose, xylose or lactose yield the lowest amounts of saadamycin. The utilization of glucose and starch by Ah22 for growth and production of the antifungal metabolite suggests the presence of an active uptake system for these substrates, as previously reported for other *Streptomycetes* species [3, 5].

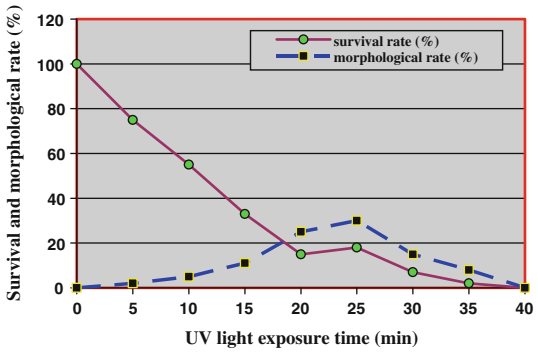

Fig. 2 Effect of UV exposure time on survival and morphological rate of *Streptomyces* sp. Hedaya48

Table 2 Highest antifungal saadamycin induced mutants

Exposure time (min)	Mutant no.	Antifungal increasing (fold)
10	Ah22	10.5
	Ah25	2.4
	Ah30	3.5
	Ah32	5.1
15	Ah38	1.9
	Ah45	4.8
20	Ah53	1.6
	Ah60	3.8
	Ah62	2.3
30	Ah69	1.2

Streptomyces sp. Hedaya48 produced 40 μg/ml saadamycin (control)

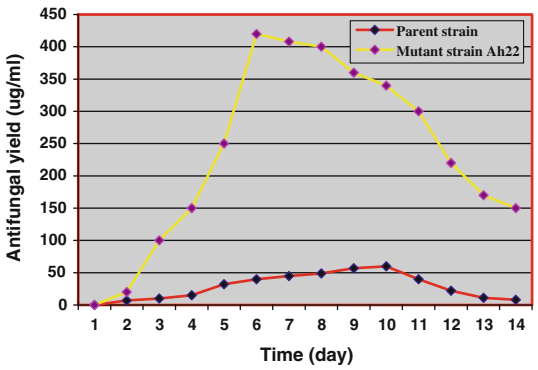

Fig. 3 Fermentation curve of antibiotic production by parent strain Hedaya48 and its mutant Ah22

Influence of nitrogen source

The results (Table 4) revealed that the level of antifungal production was greatly affected by the nature and concentration of the nitrogen source supplied in the culture medium. The antifungal production was increased by 21.5, 9.6 and 7.2% in cultures of Ah22 containing valine, alanine

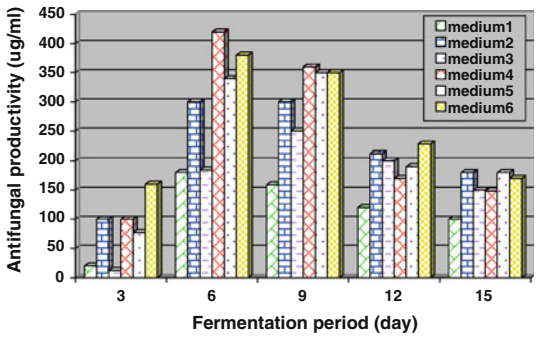

Fig. 4 Effect of different fermentation media on antifungal productivity by mutant Ah22

Table 3 Effect of different carbon sources on saadamycin production by mutant Ah22

Carbon source	Final pH	Dry weight (mg/ml)	Antifungal concentration (μg/ml)
Fructose	5.7	1.12	372
Glucose	7.8	2.12	400
Mannose	6.0	3.00	364
Galactose	7.2	0.9	10
Xylose	5.8	0.9	11
Lactose	6.4	1.0	16
Sucrose	6.8	2.12	86
Maltose	6.5	0.84	120
Starch	8.0	2.55	420
Glycerol	8.22	3.20	230
Mannitol	8.0	2.60	312
Raffinose	7.0	1.22	185
1% Starch + 0.5% glucose	8.0	3.14	450
1% Starch + 1% glucose	8.11	3.30	512
1% Starch + 2% glucose	8.2	3.00	500

or phenylalanine, respectively, as a sole nitrogen source (with 1% glucose and 1% starch as carbon source). However, cultures containing tryptophan, asparagine or ammonium salts showed the lowest antifungal activity. Cultures supplemented with a mixture of $NaNO_3$, alanine, phenylalanine and valine showed a 53.5% increase of antifungal production.

Influence of elements

The results given in Table 5 show that the antifungal yield in the presence of 0.1% KH_2PO_4, 0.05% $MgSO_4$, 0.2% $CaCO_3$, 0.1% NaCl or 0.02% $FeSO_4$ was increased by 6.6, 5.5, 10.1, 6.2 and 4.3, respectively, compared to the control (the modified starch nitrate medium with 0.1% KH_2PO_4, 0.1% K_2HPO_4, 0.05% $MgSO_4 \cdot 7H_2O$, and 0.05%

Table 4 The role of nitrogen source on the production of antifungal saadamycin by mutant Ah22

Nitrogen source	Final pH	Dry weight (mg/ml)	Antifungal concentration (μg/ml)
$NaNO_3$ (control)	8.00	3.44	512
Casein	6.3	2.0	90
Peptone	6.3	3.1	250
Yeast extract	6.5	3.5	320
Tryptone	6.5	3.12	300
Soybean	6.8	3.6	390
NH_4NO_3	5.7	1.8	80
$(NH_4)_2SO_4$	5.40	2.32	44
KNO_3	8.12	4.12	500
Glutamic acid	7.0	2.5	140
Phenylalanine	6.62	2.4	549
Alanine	6.90	2.9	561
Valine	7.42	3.0	622
Tryptophan	6.18	2.64	22
Asparagine	6.9	1.48	30
Alanine + phenylalanine + valine	7.9	4.00	650
$NaNO_3$ + alanine + phenylalanine + valine	8.5	4.22	786

KCl). There have been only a few detailed investigations on the mineral requirements during biosynthesis of antibiotics by *Actinomycetes*. Low concentrations of phosphate and ferrous ions previously were shown to support mildiomycin production by *S. rimofaciens* [14, 15]. The positive action of mineral ions may be related to their roles in formation of peptidases and proteases. We can conclude that changes in the nature, type and concentration of carbon and nitrogen sources, or the mineral element component of the culture medium greatly affect antibiotic biosynthesis in *Streptomycetes,* as previously reported by many authors [3, 5].

Influence of incubation temperature and initial pH value

Mutant Ah22 tolerated a wide range of incubation temperatures for relatively good growth and antifungal production. Maximum antibiotic production (922 μg/ml) was obtained at 35°C. Higher incubation temperature (37–45°C) had an adverse effect on growth and antifungal production. On the other hand, the initial pH value of culture medium showed a significant effect on the growth and antifungal production of Ah22 strain. The maximum growth and antifungal activity (950 μg/ml) was obtained at an initial pH of 6.5–7.0.

Finally, production medium containing (g/l) starch, 10; glucose, 10; $NaNO_3$,1.0; valine, 0.5; alanine, 0.25; phenylalanine, 0.25; KH_2PO_4, 1.0; $MgSO_4$, 0.5; $CaCO_3$, 2.0; NaCl, 1.0; $FeSO_4$, 0.2; seawater 1 l; and pH 6.5 at 35°C was recommended for saadamycin production by mutant Ah22. The optimized medium enhanced antifungal productivity to 2.26-fold (950 μg/ml) and 2.38-fold (1,000 μg/ml) in batch culture and bioreactors, respectively, compared with the yield obtained in the normal production medium (420 μg/ml; Table 6).

Structure elucidation of bioactive compounds

Chemical structures of vanillin [1]; 5,7-dimethoxy-4-p-methoxylphenylcoumarin [2] and saadamycin [3] are presented in Fig. 5. Compound 1, vanillin, did not exhibit any antifungal activity, and it was identified by comparison with authentic samples. Compound 2 (5,7-Dimethoxy-4-p-methoxylphenylcoumarin C18H16O5, Fig. 5) was a white powder: melting point, 150–152°C (EtOH); UV λ_{max} (MeOH) nm (log ε): 250 (4.07), 325 (4.29); ^{1}H NMR ($CDCl_3$, 500 MHz) δ7.20 (2H, d, J=8.5 Hz, H-2′, H-6′), 6.87 (2H, d, J=8.5 Hz, H-3′, H-5′), 6.50 (1H, d, J=2.5 Hz, H-8), 6.22 (1H, d, J=2.5 Hz, H-6), 5.96 (1H, s, H-3), 3.83 (6H, s, OMe-7, OMe-4), 3.46 (3H, s, OMe-5); IR ν_{max}($CHCl_3$) cm^{-1}: 1,710, 1,610, 1,595, 1,510, 1,158, 1,111, 1,052, 952, 872, 860, 830; MS *m/z* (relative intensities): 312 $[M]^+$ (80), 284 $[M–CO]^+$ (100), 269 $[M–MeCO]^+$ (37), 241 $[M–43–CO]^+$. The identification of this compound was confirmed by comparison with authentic sample.

Structure determination of the new antimycotic compound, saadamycin

The physico-chemical properties of ^{13}C NMR and ^{1}H NMR and HMBC data of saadamycin are illustrated in Tables 7, 8 and Figs. 5, 6. Saadamycin antibiotic was obtained as yellowish white powder. High-resolution ESI-MS revealed a molecular ion peak at m/z 141.0183 for $[M-H]^-$ (calcd. 141.0188) corresponding with the molecular formula $C_6H_6O_4$ with four degrees of unsaturation. The IR spectrum absorptions at 1,615 and 3,176 cm^{-1} indicated the presence of carbonyl and hydroxyl functional groups. The NMR spectra revealed the presence of one oxygenated aliphatic methylene carbon (δ_C 59.5), two olefinic methine carbons (δ_C 109.8, 139.2), one enolic carbon (δ_C 168.1), one quaternary olefinic carbon (δ_C 145.7) and a carbonyl carbon (δ_C 173.9). These data indicated the presence of a hydroxy-substituted 2-pyrone ring in the saadamycin structure. The presence of the 4-hydroxymethyl group was detected by the ^{1}H -NMR [4.29 (2H, s), 5.67 (1H, s)]. Additionally, HMBC correlations (Fig. 6) from δ_H 6.34 (1H, s, H-3) to C-2, C-4, C-5 and C-7, from δ_H 8.03 (1H, s, H-6) to C-2, C-4 and C-5, and from δ_H 4.29 (2H, s, H-7) to

Table 5 The role of trace elements on the production of antifungal saadamycin by mutant Ah22

Element	Recommended concentration (%)	Final pH	Dry weight (mg/ml)	Antifungal concentration (μg/ml)
Control	–	8.39	4.00	786
$MgSO_4$	0.05	6.3	2.0	829
$FeSO_4$	0.02	6.62	3.1	820
$MnCl_4$	0.01	6.50	3.5	500
$ZnSO_4$	0.02	6.00	3.12	540
$CuSO_4$	0.01	6.51	3.6	340
KCl	0.01	6.70	1.8	770
NaCl	0.1	7.82	3.72	835
$CaCO_3$	0.2	6.90	3.64	865
K_2HPO_4	0.2	8.12	4.12	650
KH_2PO_4	0.1	7.22	3.50	838
$KH_2PO_4 + MgSO_4 + CaCO_3 + NaCl + FeSO_4$	–	7.60	3.39	900

Table 6 Effect of culture conditions on growth and antifungal production by the parent and mutant Ah22 strain

Culture condition	Dry weight (mg/ml)		Antifungal concentration (μg/ml)	
	Parent	Mutant Ah22	Parent	Mutant Ah22
Normal medium	3.22	2.89	40	420
Optimized medium	5.8	3.32	100	950
Optimized medium (bioreactor)	7	6.8	120	1,000

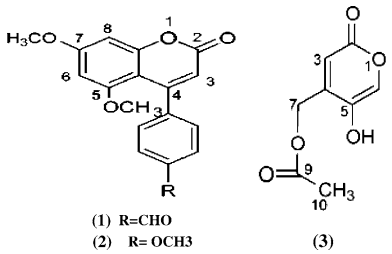

Fig. 5 Chemical structures of vanillin (*1*); 5,7-dimethoxy-4-p-methoxylphenylcoumarin (*2*) and saadamycin (*3*)

Table 7 Physico-chemical properties of saadamycin antibiotic

Appearance	Yellowish white
Melting point	140–142°C
HRESI-MS *m/z*	141.0183 for (M-H)—calcd. 141.0188 for $C_6H_5O_4$
Molecular formula	$C_6H_6O_4$
UV λ_{max} (MeOH) (log ε)	213 (4.75), 273 (3.90) nm
IR (KBr) ν_{max}	3,258, 3,176, 1,615, 1,460, 1,283 cm^{-1}

Table 8 ^{1}H- and ^{13}C-NMR, HMBC assignment of saadamycin in DMSO-d6, 500 and 125 MHz, TMS and δ in ppm

Number	δ_H [*J* in Hz]	δ_C	HMBC
2	–	173.9 s	2, 4, 5, 7
3	6.34 (1H, s)	109.8 d	–
4	–	145.7 s	–
5	–	168.1 s	2, 4, 5
6	8.03 (1H, s)	139.2 d	3, 5
7	4.29 (2H, s)	59.5 t	6
5-OH	9.07 (1H, s)	–	–
7-OH	5.67 (1H, s)	–	

Fig. 6 HMBC correlations observed saadamycin antibiotic

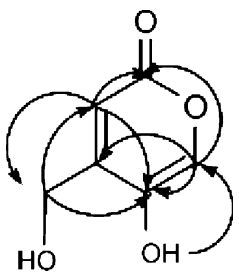

Table 9 Minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) values of the bioactive compounds and myconazole against dermatophytes and other clinical fungi

Test organism	Saadamycin (μg/ml)		5,7-Dimethoxy-4-*p*-methoxylphenyl coumarin (μg/ml)		Myconazole (mg/ml)	
	MIC	MFC	MIC	MFC	MIC	MFC
Trichophyton rubrum	5	10	7.5	100	6	12
Trichophyton mentagrophytes	1.5	1.75	90	90	3	6
Microsporum gypseum	1.25	1.25	100	150	3	6
Epidermophyton floccosum	1.0	1.5	50	66	6	12
Aspergillus niger	1.0	1.6	20	50	10	22
Aspergillus fumigatus	1.6	2.0	10	35	12	15
Fusarium oxysporum	1.2	2.0	22	49	8	12
Candida albicans	2.22	3.0	15	20	20	20
Cryptococcus humicolus	5.16	5.55	10	32	10	15.5

C- 3, C-5 revealed the structure of compound 3 as 4-(hydroxymethyl)-5-hydroxy-2*H*-pyran-2-one.

MIC and MFC values of saadamycin from mutant Ah22 against dermatophytes

The lower MIC (1–5.16 μg/ml) and MFC (1.25 to10 μg/ml) values of saadamycin antibiotic, and MIC (7.5–100 μg/ml) and MFC (20 to150 μg/ml) values of 5,7-dimethoxy-4-pmethoxylphenylcoumarin compared favorably to myconazole (MIC, 3–20 and MFC 6–22 mg/ml, Table 9), indicating to the efficiency of these bioactive metabolites of Ah22 against dermatophytes and other pathogenic fungi. Bioactive pyrone derivatives were previously isolated from marine *Streptomyces* and shown to be potent antimicrobial agents against some pathogenic bacteria and fungi [6]. On the other hand, previous reports have indicated that 5,7-dimethoxy-4-pmethoxylphenylcoumarin was produced by numerous species of plants [19], but to our knowledge this is the first report of its production from a microbial source as well as its antimycotic activity. Our study is the first in which saadamycin and 5,7-dimethoxy-4-p-methoxylphenylcoumarin were isolated from culture filtrates of an endophytic *Streptomyces* species of Egyptian sponge *Aplysina fistularis*.

Conclusion

The results of this study conclude that saadamycin antibiotic is a major ingredient in the culture filtrate of *Streptomyces* sp. Hedaya48, and it can play an important role in the inhibition of dermatophytes and other fungal pathogens. Further investigations are necessary to determine the relationship between these bioactive compounds and the host sponge through this intimate interaction.

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