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Isolation and characterization of an antifungal agent active against human pathogenic fungi, produced by *Pseudomonas fluorescens* FSJ-3

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عزل و تحديد بنية نشاط المواد المضادة المحضرة ب Pseudomonas fluorescens FSJ 3

ثم عزل درية FSJ 3 دات نشاط قوي مضاد للفطور، و ذلك بمنطقة فاس (المغرب). و تم استخراج المادة النشيطة من وسط زراعي سائل محلى (وسط Sabourand) و قد تم تحليل الفينازينيكاربوميد المحصل عليه على شكل زجاج أصفر، و ذلك عن طريق RMN-^{1}H و ^{13}C بطريقة سبيكترومتري. و هذه الجزيئة توقف تكاثر عدد الباكتيريا و الخميريات و الفطر المليفة ك :Candida albicans, Cryptococcus neoformans, Aspergillus fumigatus. و تحدث الفينازيكاربوميد تغير مورفولوجي لـ Arthroderma simii ، كما أنها ليست سامة لكُرَيْفاوة الفئران ب (200Ug/ml) من التركيز.

الكلمات المفتاحية : Pseudomonas fluorescens –مواد مضادة للفطور– الفينازيكربوميد – فاس – المغرب.

Isolement et caractérisation d'une substance antifongique élaborée par *Pseudomonas fluorescens* FSJ-3, active contre des champignons pathogènes de l'homme

La souche FSJ-3 de *Pseudomonas fluorescens* douée d'une forte activité antifongique contre les champignons pathogènes de l'homme a été isolée à partir du sol (Fès, Maroc). La substance active, élaborée par cette souche, a été extraite du milieu de culture liquide, avec du n-butanol et purifiée par chromatographie sur gel de filtration et sur gel de silice. Le 1-phénazinecarboxamide, obtenu sous forme de cristaux jaunes, a été analysé par RMN ^{1}H et ^{13}C et par spectrométrie de masse. Cette molécule inhibe la croissance de plusieurs bactéries, levures et champignons filamenteux, comme *Candida albicans, Cryptococcus neoformans* , *Aspergillus fumigatus*. Le 1-phénazinecarboxamide provoque des altérations morphologiques sur les hyphes d' *Arthroderma simii*. À forte concentration (200 µg/ml), il n'est pas toxique pour les leucocytes de rat.

Mots clés : *Pseudomonas fluorescens* - Substance antifongique - 1-phénazinecarboxamide -Champignons pathogènes - Sol

Isolation and characterization of an antifungal agent active against human pathogenic fungi, produced by *Pseudomonas fluorescens* FSJ-3

An antifungal agent, produced by *Pseudomonas fluorescens* strain FSJ-3 originating from Moroccan soil (Fès), was isolated and characterized. It was extracted from Sabouraud's glucose broth culture by *n*-butanol and purified by gel filtration and silica gel chromatography giving yellow crystals. Its structure was assigned to 1-phenazinecarboxamide by analysing ^{1}H and ^{13} C-NMR and mass spectral data. It showed excellent activity against several species of bacteria, yeasts and human pathogenic filamentous fungi , including *Candida albicans*, *Cryptococcus neoformans, Aspergillus fumigatus* and caused morphological modifications on *Arthroderma simii* hyphae. 1-phenazinecarboxamide was non toxic when tested on rat leucocytes up to the highest concentration (200 µg/ml).

Key words : *Pseudomonas fluorescens* - Antifungal antibiotic - 1-Phenazinecarboxamide - Pathogenic fungi - Soil

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INTRODUCTION

Several antimicrobial substances produced by *Pseudomonas* species have been found. The nature of these products was variable (Katayama *et al.*, 1993 ; Kintaka *et al.* , 1981 ; Kintaka. *et al.*, 1984 ; Shoji *et al.*, 1990). Among them phenazine compounds (Gurusiddaiah*et al.*, 1986 ; Jones *et al.* ,1988 ; Kanner *et al.* , 1978) such as phenazine-1-carboxylic acid and 1-phenazine-carboxamide. The activity of these substances against bacteria and phytopathogenic fungi has been reported (Gurusiddaiah *et al.*,1986). On the contrary, little is known about the activity of these agents against zoopathogenic fungi.

This paper deals with the production, isolation and activity against yeast and human pathogenic fungi of the antifungal agent, produced by*Pseudomonas fluorescens* strain FSJ-3 isolated from Moroccan soil.

MATERIALS AND METHODS

1. Production of the antifungal agent

For production of antifungal compounds, the *Pseudomonas fluorescens* FSJ-3 strain was isolated from a soil sample collected in Fes city, (Morocco), and grown on Sabouraud's glucose broth (Peptone: 5 g; glucose 20 g; caseine hydrolysate 5 g; distilled water; pH 5.8). The culture medium (2.5 l) was placed in five erlenmeyer flasks, containing each 500 ml of broth and autoclaved at 120°C for 15 min. After autoclaving the flasks were inoculated with 50 ml of two days old preculture of *P. fluorescens* and incubated at 30°C on a shaker (Lab. Shaker Adolf Kuhner Ag Schweib) at 90 rpm for 10 to 12 days.

2. Biological assays

Antifungal activity of the crude supernatant and of the different fractions obtained after each purification step was determined by microtechniques (Mor *et al.* , 1993) : 10 ml Sabouraud's glucose agar (at 45°C) containing 10^4 of yeasts or 10^5 fungal spores suspension were poured over a Petri dish (diam 90 mm) and allowed to harden at room temperature. Round (diam. 4 mm) or square (4x4 mm) slices were cut, deposited on one of the eight circles of an immunofluorescent Microprint slide and submerged in 10 µl of crude *P. fluorescens* FSJ-3 filtrate. The microculture was incubated at 30°C. The inhibition of cellular multiplication, spore germination and hyphal elongation and the morphological alterations were observed in a light microscope. The minimal inhibitory concentration (MIC) of the purified antimicrobial substances against bacteria, yeasts and several filamentous fungi were determined by a microplate automatized technique (Drouhet *et al.*, 1986) (Nunk F 96 microtiter plates, Roskild, Denmark).

The antimicrobial assays were performed in sterilized 96 well plates in a final volume of 100 ml. The Sabouraud's glucose liquid medium (100 ml) containing the antifungal agent in serial two fold dilution, 100 µl 0.4 % formol/water as negative control or without added antifungal agent as positive control was distributed with a multipipet. 10 µl of bacterial suspension (10^8 cells/ml) , yeasts (10^5/ml) or spores (10^6/ml) in the appropriate culture medium (LB medium for bacteria, Sabouraud's glucose broth for yeast and fungi) were added to each well. Growth inhibition was determined by measuring the optical density at 492 nm with a Titertek Multiskan Mcc after 48 hours or 72 hours of incubation at 30°C for yeasts and fungi or 37°C for bacteria.

The bioautography technique was used for composition analysis of crude extract and antifungal agent localisation. Aliquotes of each fraction (1 to 5 ml) were loaded on silica gel thin layer chromatography (TLC). The chromatograms were developed in appropriate solvent. These plates were dryed and antifungal substances were detected by depositing the TLC plates on Sabouraud's glucose agar Petri dishes including the indicator strain (*Arthroderma simii* 10^5 spores/ml). After prediffusion at room temperature, inoculated plates were incubated at 30°C for 48 hours. The inhibitory fraction was detected by showing clear zone arround corresponding spot. Its Rf was then measured.

The cytotoxicity of 1-phenazinecarboxamide was assayed by monitoring the permeability of rat polymorphonuclear leucocytes (10^6 Cells/ml) to trypan blue (0,1 g/l). After 10 minutes of incubation in the presence of antifungal substance, dead cells (coloured) were counted. The substance was considered as nontoxic when cell viability is more than 95%.

3. Isolation of the antifungal agent

P. fluorescens FSJ-3 culture broth was centrifuged at 3500 rpm. The supernatant was extracted twice

with *n*-butanol (1 l). After separation of the aqueous and organic phases, the inactive aqueous phase was discarded, and the active butanol phases were combined and the solvent removed under reduced pressure. The residue (7.25 g) was submitted to gel chromatography on Sephadex LH 20 (67 x 2 cm) with methanol as eluent. The fractions were collected and submitted to antifungal tests. The active fractions were pooled and chromatographed on a silica gel column (50 x 2 cm), eluted with methylene chloride (500 ml), and with methylene chloride/methanol (95:5) and (90:10). Crude 1-phenazinecarboxamide (PZC) (50 mg) was eluted with methylene chloride/methanol (90:10). It was further purified by silica gel chromatography (20 x 1 cm) and eluted with *n*-hexane /EtOAc (1:1).

4. General methods

Melting point was uncorrected. Mass spectrum was taken under electron impact (70 eV) using direct inlet sample introduction on a Kratos MS 80 spectrometer. ^{1}H (300.13 MHz) and ^{13}C (75.47 MHz) NMR spectra were performed for a 25 mM solution in $CDCl_3$ on a Bruker AM 300 spectrometer equipped with an Aspect 3000 computer. ^{1}H and ^{13}C chemical shifts were referenced to tetramethylsilane (TMS).

5. Thin layer chromatography

The purification steps and the homogeneity of the isolated compounds were monitored by silica gel thin layer chromatography (TLC) with the following systems: silica gel G 60 F 254 (Merck 5554); *n*-butanol, acetic acid, water: 6/2/2 (BAW) or methylene chloride/MeOH: 9/1 (MCM). The plates were visualized either by UV (235 and 265 nm) or by spraying with anisaldehyde reagent (acetic acid, sulfuric acid and *p*-anisaldehyde : 25/1/1) and heating (120°C). The Rf of 1-phenazine-carboxamide was in BAW : 0.86 and in MCM : 0.91.

6. 1-Phenazinecarboxamide properties

Yellow crystals (m.p: 248°C) soluble in chloroform, dimethylsulfoxide, insoluble in methanol and water. EIMS: *m/z* (rel. int) : $[M]^+$ 223 (67); 205 (40); $[M-NH_2]^+$ 207 (23) ; $[M-CONH_2]^+$ 180 (100) $[M-CONH_2]^+$ 179 (40); 152 (123); 129 (5); 112 (3); 102 (13); 90 (12); 76 (19).

^{1}H NMR ($CDCl_3$, 300.13 MHz), δ (ppm), J (Hz) : 10.68 (1H, bs, $CONH_2$ *anti*); 8.99 (1H, dd, 7.1, 1.3, H-2); 8.40 (1H, dd, 8.7, 1.3, H-4); 8.27-8.18 (2H, H-6, H-9); 7.94 (1H, dd, 8.7, 7.1, H-3); 7.90-7.87 (2H, H-7, H-8); 6.41 (1H, bs, CONH *syn*).

^{13}C NMR ($CDCl_3$, 75.47 MHz), δ (ppm) : 166.6 (C=O) , 143.4 (C_4 a), 143.1 (C_5 a #), 141.5(C_9 a #), 140.8 (C_{10} a), 135.9 (C_2), 134.3 (C_4), 131.7 (C_8*), 131.1(C_7*) 129.9 (C_3),129.7(C_9§), 129.1 (C_6 §), 128.9 (C_1); assignement with, #, *, and § may be reversed.

RESULTS AND DISCUSSION

• Extraction and purification of 1-phenazinecarboxamide

After incubation at 30°C for 12 days , the culture of *P. fluorescens* FSJ-3 was centrifuged and filtered, and the culture broth was extracted two times with *n*-butanol. The organic extract exhibited antifungal activity, whereas no such activity was detected in the aqueous phase. The organic extract was thus fractionated as described in figure 1. We obtained 35 mg of pure 1-phenazinecarboxamide. Through the purification steps, aliquotes from each fraction were tested for antifungal activity. Fractions containing antifungal agent showed strong growth inhibition against *Cryptococcus neoformans* and *Arthroderma simii* as suggested by their respective MIC's.

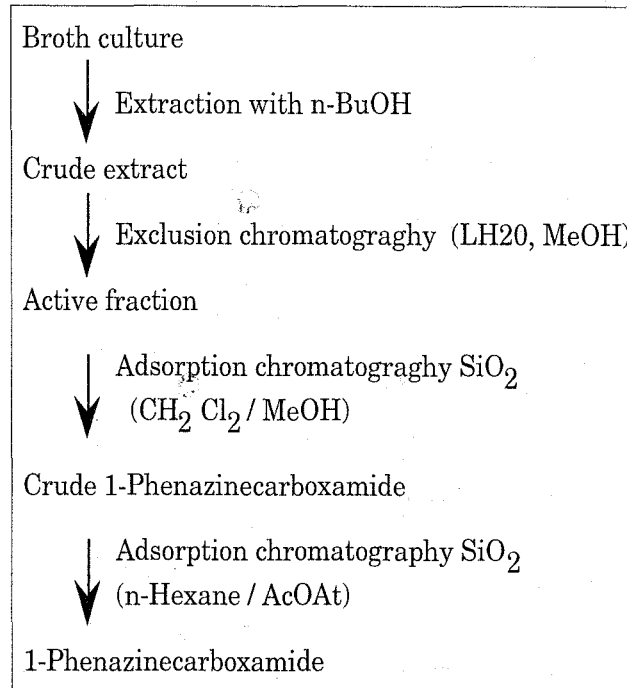

Figure 1. Isolation and purification of 1-phenazine-carboxamide from *Pseudomonas fluorescens* FSJ-3

- **Spectroscopic characterization of 1-phenazinecarboxamide**

Several spectroscopic studies (IR, ^{1}H and ^{13}C NMR) of antibacterial phenazine compounds have been reported in the literature (Breitmair & Hollestein , 1976 ; Gurusiddaiah *et al.,* 1986 ; Romer, 1982 &1983 ; Stammer & Taurins,1963), but spectroscopic data on 1-phenazinecarboxamide (PZC) are less documented.

EI mass spectrum of 1-PZC exhibited the molecular ion at m/z 223 and a fragmentation at 207 characterising the loss of the amino group. The peak at *m/z* 179 indicated the loss of carboxamide group; it was accompanied by the base peak at *m/z* 180 which indicated a rearrangement due to the capture of hydrogen to complete the phenazine ring. This had been previously observed by Kanner *et al.* (1978).

The ^{1}H NMR spectrum ($CDCl_3$) exhibited two broad singlets at 10.68 and 6.41 ppm representative of the *anti* and *sym* protons of the carboxamide group, respectively. The monosubstituted phenazine ring showed the characteristic pattern: three resonances appeared as doublets of doublets at d 8.98, 8.40 and 7.94 corresponding to H_2, H_4 and H_3 respectively, and consistent with the carboxamide substituent effect in benzene.

The chemical shift of H_2 was unambiguously assigned from observation of it $^{3}J_{C\text{-}H}$ coupling to the carboxamide CO group in the HMBC spectrum. The resulting assignement of H_2 and H_4 are reversed, as compared to that found in the literature (Gurusiddaiah *et al.,* 1986 ; Romer, 1982).

The remaining protons belonging to the unsubstituted aromatic ring exhibited the ABCD pattern of the phenazine rings, wich H_6 and H_9 between 8.27 - 8.18 ppm and H_7, H_8 between 7.90 - 7.87 ppm, in agreement with previous studies on 1-phenazine carbomethoxy (Gurusiddaiah *et al.,* 1986 ; Romer, 1982). Data are given in materials and methods.

1-phenazinecarboxamide was the subject of different ^{13}C NMR studies (Breitmair & Hollestein , 1976 ; Romer, 1983). The assignements resulting from the two studies were different. Therefore we assigned the ^{13}C NMR spectrum of 1-phenazinecarboxamide from the HMBC spectrum. Nevertheless, due to overlapping of some ^{1}H resonances, some of the assignements may be reversed (see materials and methods).

A controversy occured in the literature concerning the structure elucidation of the closely related 1-phenazinecarboxylic acid. A dimeric structure had been proposed by Gurusiddaiah *et al.* (1986), whereas Brisbane *et al.* (1987) provided evidence for the monomeric structure, which was definitely accepted from unequivocal evidence of the crystal structure (Romer, 1983).

The spectroscopic data presented here are in favour of the monomeric structure of the isolated antifungal agent (Figure 2).

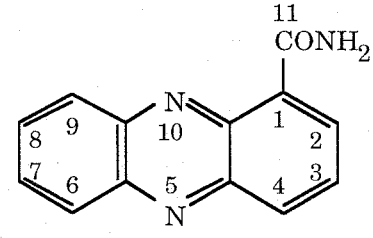

Figure 2. Structure of 1-phenazinecarboxamide isolated from *P. fluorescens* FSJ-3

- **Biological properties of 1-phenazine-carboxamide** (1-PZC)

The spectrum of antimicrobial activity of 1-PZC is presented (Table 1) in terms of minimal inhibitory concentrations (MIC) giving 100 % inhibition of the microorganisms tested, including Gram-positive and Gram-negative bacteria, yeasts and filamentous fungi.

However, various microorganisms exhibited different degrees of sensitivity, as indicated by the MICs in the range 0.3-100 µg/ml. The antibiotic showed strong activity against bacteria (such as *Entercoccus faecalis*) and several fungi, particularly dermatophytes. It exhibited also moderate activity against other bacteria and yeasts. The 1-PZC inhibited spore germination and growth inhibition of *A. simii* and caused morphological modifications in hyphea (Figure 3).

In growth inhibition assays performed on mycelium of *A. simii*, no visible hyphae elongation could be seen in treated sample, whereas untreated sample developed long hyphae. Observation of mycelium in light microscope indicated clearly that the hyphae of *A. simii* were altered. The cytoplasm was contracted and empty spaces appeared in

fungal cells and sometimes protoplasm ejection were noted. Different data (non reported here) suggets that the FSJ-3 strain of *P. fluorescens* produce minor antibiotics that also exhibit antifungal activity.

These products could probably be minor phenazines types since *Pseudomonas* sp was reported to produce more than one phenazine (Chang & Blackwood, 1969 ; Kanner *et al.*, 1978, Toohey, 1965).

1-phenazinecarboxamide produced by *Pseudomonas fluorescens* FSJ-3 did not induce obvious damage to rat leucocytes *in vitro* and did not affect the permeability of leucocytes up to the highest concentration assayed (200 µg/ml). Gurusiddaiah *et al.* (1986) reported that a dimer phenazinecarboxylic acid was no toxic to mice receiving up to 464 mg/kg of the antibiotic by oral route.

Table.1. Spectrum of antimicrobial activity of 1-phenazinecarboxamide reported in terms of minimal inhibitory concentration (MIC)

Test organisms	Strain	MIC (µg/ml)
Pseudomonas aeruginosa	**	100
Enterococcus faecalis	IP 103214	0,39
Staphylococcus aureus	IP 76-25	50
Cryptococcus neoformans	IP 960	50
Candida albicans	IP 88465	100
Trichophyton rubrum	IP 2043	12,5
Trichophyton mentagrop	IP 1468	12,5
Arthroderma simii	IP 90265	25
Arthroderma benhamiae	IP 1064	12,5
Microsporum canis	IP 1194	3,12
Aspergillus niger	IP 21853	12,5
Aspergillus fumigatus	IP 1025	100

IP: Institut Pasteur of Paris (France)
** Strain isolated from onycomycosis patients

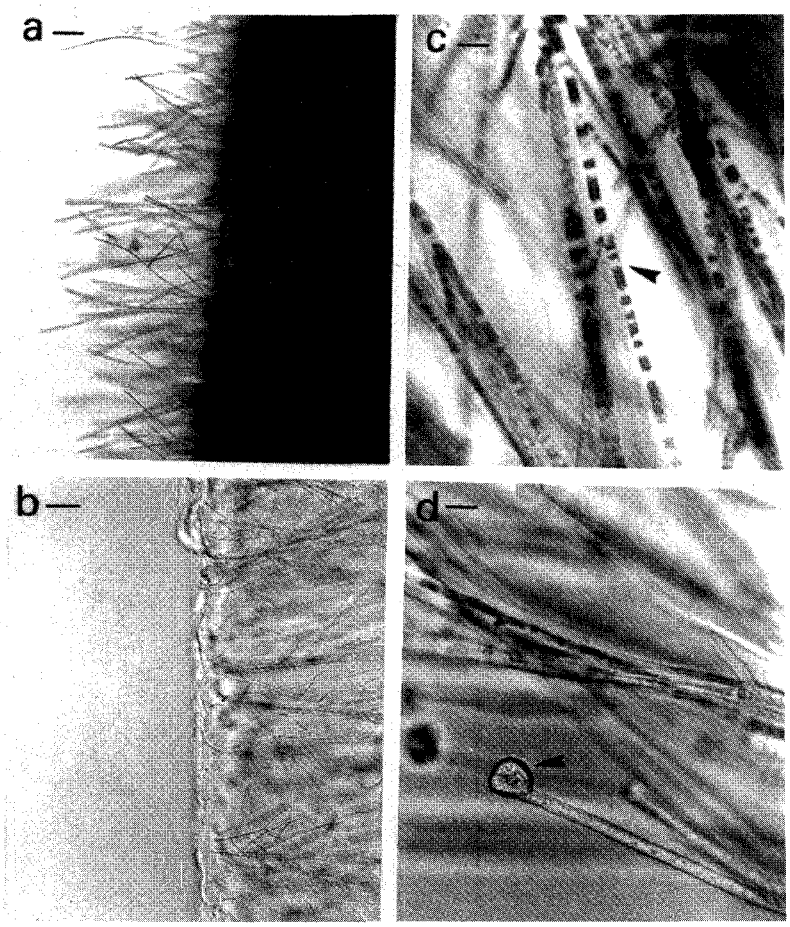

Figure 3. Hyphal elongation inhibition and morphological effects of 1-phenazinecarboxamide on *A. simii*
a: control: elongation hyphae
b: inibition of hyphae elongation
c: cytoplasmic condensation
d: protoplasm ejection
Bar equals: 50 mm.

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