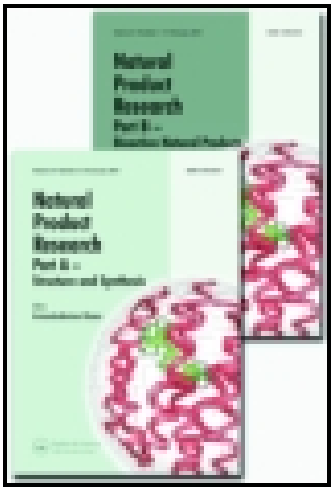

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Penicillium commune metabolic profile as a promising source of antipathogenic natural products

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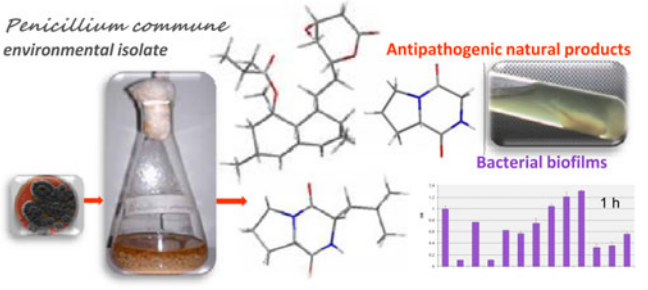

Penicillium is an important genus of ascomycetous fungi in the environment and in food and drug production. This paper aims to investigate statins and antipathogenic natural products from a *Penicillium commune* environmental isolate. Fractions (F1, F2, F3 and F4) were obtained from an ethyl acetate extract. Direct insertion probe/electron ionisation/ion trap detection mass spectrometry (MS and MS/MS) identified lovastatin (**1**) in F1, while GC-MS showed that 3-isobutylhexahydropyrrolo[1,2-a]pyrazine-1,4-dione (**2**) was the main constituent of F2 (49.34%). F4 presented **3** (16.38%) as an analogue of **2** and their known structures were similar to that of an autoinducer-signal. F1 produced a significant decrease in the *Pseudomonas aeruginosa* biofilms, which is the main cause of bacterial pathogenicity. F2 and F4 were effective against *Staphylococcus aureus* biofilms, but when F2 was associated with oxacillin, it showed an important activity against both bacteria. These novel results suggest that *P. commune* INTA1 is a new source of promising antipathogenic products.

Keywords: *Penicillium commune* metabolic profile; DIP/EI/ITD mass spectrometry (MS and MS/MS); GC-MS analysis; lovastatin; pyrrolopyrazines; biofilms

1. Introduction

Penicillium is a genus of ascomycetous fungi of major importance in the natural environment and in food and drug production. They are among the most common fungi that spoil food and contaminate indoor environments (Samson et al. 2004). Among fungal metabolites, statins are a group of pharmacological interest as hypocholesterolemic drugs. According to Endo (2004), the

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fungal production of statins would occur as a defence mechanism against other microbes that require sterols and/or other mevalonate-derived isoprenoid compounds for their growth. In fact, statins inhibit HMG-CoA reductase, the enzyme that plays a central role in cholesterol production. Increased cholesterol levels have been associated with pathological conditions like atherosclerosis and cardiovascular diseases. Statins are therefore used in their prevention. Mevastatin was first isolated from *Penicillium citrinum* broths. Lovastatin (**1**) was discovered next from soil fungus *Aspergillus terreus*, xerophilc fungus *Monascus ruber*, species of the genera *Hypomyces*, *Doratomyces*, *Phoma*, *Eupenicillium*, *Gymnoascus* and *Trichoderma*, and recently from the plant pathogen *Penicillium funiculosum.* The other statins were obtained by a semi-synthetic process, involving the chemical modification of the lovastatin side chain, as in the case of simvastatin, or by the biotransformation of mevastatin by *Streptomyces carbophilus* like pravastatin. Finally, fluvastatin and atorovastatin are fully synthetic statins, derived from mevalonate and pyridine, respectively (Manzoni & Rollini 2002; Endo 2004; Sri Rami Reddy et al. 2011).

Bacteria are able to coordinate gene expression as a community through the secretion and detection of signalling molecules so that its members can simultaneously express-specific behaviours. This mechanism of behaviour regulation appears to be a key trait for adaptation to specific environments and has been shown to control a variety of important phenotypes, like the production of biofilm. The ability to communicate and communally regulate gene expression is hypothesised to have evolved as a way for organisms to delay expression of phenotypes until numerical supremacy is reached. In case of infection, if an invading microorganism were to express virulence factors too early, the host may be able to mount a successful defence to repel the invaders. There is growing evidence that bacterial quorum sensing (QS) systems are involved in cross-kingdom signalling with eukaryotic organisms and that eukaryotes are capable of actively responding to bacteria in their environment by detecting and acting upon the presence of these signalling molecules. Likewise, eukaryotes, such as fungi, produce compounds that can interfere with QS systems in bacteria by acting as antagonists. A new field of study takes inspiration from nature's models and attempts to design solutions for infection problems (McDougald et al. 2007). Microbial biofilms consist of groups of bacterial cells adherent to a surface and enclosed within a self-produced extracellular matrix. Adaptation to surface-attached growth within a biofilm is accompanied by significant changes in gene and protein expression, as well as metabolic activity which confers resistance to environmental toxins and antimicrobial therapy. Bacterial biofilm phenotype is a major virulence factor contributing to the chronicity of infections (Sanchez et al. 2013).

The present research is focused on determination of statins and antipathogenic natural products from complex matrices of an environmental isolate of *Penicillium commune* INTA1. In addition, their anti-biofilms effects against two pathogenic bacteria of *Staphylococcus aureus* and *Pseudomonas aeruginosa* with biofilm phenotypes are reported for the first time in this research.

2. Results and discussion

2.1. *Optimal culture conditions for ubiquitous* P. commune *fungus*

Filamentous fungi have been extensively used in biotechnological processes such as cell factories due to the metabolic versatility of this group of microorganisms. They are able to produce important metabolites including many antibiotics and cholesterol-lowering drugs (statins) into submerged culture during the secondary phase (idiophase) of fungi growth. The β-hydroxyl statins are the most active form of these metabolites, but they are unstable (Gupta et al. 2007; Jaivel & Marimuthu 2010). Therefore, the fermentation conditions were optimised (28°C and pH = 6.3) according to Kumar et al. (2000), who demonstrated that this incubation

temperature and pH (between 5.8 and 6.3) provided optimum conditions for lovastatin production by *A. terreus* in the batch process. In addition, the mycelial pellet formation inside a submerged culture is observed (Supplementary Figure S1A – online only). This morphological characteristic of growth has a much larger specific surface area which reduces the mass transfer limitations; a beneficial effect on broth rheology, which in return affects momentum, mass and heat transfer in the reactor. Consequently, efficiency of mixing, aeration and cooling systems is improved. Another advantage of fungal pellet fermentation is that the pellets make it possible to perform high biomass concentration cultures to increase productivity (Liu et al. 2006).

2.2. *Extraction and isolation of main metabolites from* P. commune *INTA1*

The fungus was extracted with acetate ethyl from a fungal culture broth and spherical pellets as the lactone form. It is normally the primary lovastatin detected in the fermented products because the hydroxyl form of lovastatin is not stable. The extraction yields were $1.56\,g\,L^{-1}$ (E) and $0.076\,g\,L^{-1}$ from the *P. commune* culture containing only 50% glucose in the culture medium (E2). For this reason, the extraction process yield decreased by 20 times. This would suggest that there was a biomass reduction that resulted in a metabolites biosynthesis decrease.

The chromatographic separation of the acetate ethyl extract from *P. commune* (87.7 mg) gave four fractions that were collected according to chromatographic profiles. The F1 fraction was eluted with $CHCl_3$–AcOEt 85:15 (yield: 3.53% in relation to processed extract), and it was isolated as white needle crystals. F2 was eluted with $CHCl_3$–AcOEt 75:25 (3.76%); F3 with $CHCl_3$–AcOEt 60:40 (7.3%) and finally F4 with pure AcOEt (19.3%). F2-4 gave off a peculiar aroma, and their main constituents were analysed by GC-MS.

2.3. *Analytical thin layer chromatography*

P. commune extracts (E and E2), F1, and standards of simvastatin, lovastatin (Merck®, Darmstadt, Germany) and mevastatin (MP Biomedicals LLC®, Solon, OH, USA) were chromatographed by thin layer chromatography (TLC) using $CHCl_3$–AcOEt 4:3 (57:43) as a good developing solvent. The eluted statins were visualised under UV light at 254 nm, and when the plates were sprayed with Godin reagent, violet spots were determined with R_f similar to lovastatin standard ($R_f = 0.41$). These spots were absent in the extract from the broth culture (BC; Figure S1).

2.4. *Fourier transformed infrared spectroscopy*

Fourier transformed infrared (FT-IR) spectra were measured to determine the functional groups of statins. Only *P. commune* extracts, and F1, exhibited infrared absorption bands at 3400 (O—H st), 1750 (C═O st) and 1280 (C—O st) cm^{-1} clearly assignable to hydroxyl and ester functionality, respectively. It is important to note that typical δ-lactone absorptions at 1735 (C═O st) and $1180\,cm^{-1}$ (C—O st) were detected. These signals were absent in the spectrum of the BC extract (negative control).

2.5. *DIP/EI/ITD mass spectrometry and complementary analysis*

A good correlation between the extract mass peaks, F1 and lovastatin standard in the molecular ion region (molecular ion, adducts, isotope ions, and MH–H_2O) was found by direct insertion probe/electron ionisation/ion trap detection (DIP/EI/ITD) mass spectrometry (Figures S2 and S3a). The base peak (100% relative abundance) in various samples was $[M + H]^+$ ion, at *m/z* 405 for lovastatin and at *m/z* 419 for simvastatin, as can be observed in Figure S2. This intense peak corresponds to the protonated molecular ion or adduct ion represented by $[M + H]^+$,

formed by the interaction of a proton with the lovastatin molecule (that contains electronegative atoms or proton acceptor). This phenomenon, called self-chemical ionisation, can occur in the ion trap mass spectrometer (McLuckey et al. 1988; Pannell et al. 1989), and was observed for statin standards under the conditions of this experiment.

The smallest amounts of **1** that generate the quasi-molecular ion (*m/z* 405) were also determined. They ranged from 760 (relative intensity = 0.5) to 1444 ng (relative intensity = 1). The $[M + H]^+$ relative intensity (with respect to base peak) was also 1 for the interval of 1444–2052 ng, and only the molecular ion at *m/z* 404 ($C_{24}H_{36}O_5$) and its mass fragmentation peaks were observed at or below 420 ng.

Lovastatin (**1**) identification in F1 from *P. commune* INTA1 was achieved by the completely unambiguous correspondence between their main mass peaks and EI-MS fragmentation pathway of the lovastatin standard (Merck®). The fragment ion $[C_{12}H_{13}]^+$ at *m/z* 157 was the most abundant under the conditions of this experiment, as shown in Figure S3a. In addition, MS/MS (MS^2) experiments from the precursor $[M + H]^+$ ion generated similar ions in F1 and lovastatin (Figure S3b).

The specific optical rotation of F1 was also measured to confirm the stereoisomer biosynthesised by fungus (logically, it is the bioactive stereoisomer), and as expected it was identical to that of the lovastatin standard (Merck®, $[\alpha]_D^{25} = +323°$, 5 mg mL^{-1} in acetonitrile). In agreement with these results, absorption bands clearly assigned to the hydroxyl and ester functionalities of statins were characterised by FT-IR spectroscopy (according to 2.4 results).

Moreover, lovastatin occurrence in F1 was corroborated by RP–HPLC using a lovastatin standard (data no shown). It is important to note that RP–HPLC is suitable for the efficient screening of strains for lovastatin production, and possibly also for the determination of lovastatin in complex biological fluids or in solid state (Kysilka & Kren 1993; Jaivel & Marimuthu 2010).

Here, a quick and effective tool was employed to identify lovastatin from complex matrices such as the *P. commune* INTA1 extract and F1 using DIP/EI/ITD mass spectrometry and statin standards.

2.6. *Metabolic profile from the wild* P. commune *INTA1 strain*

GC-MS (EI) analysis of odorant F2 and F3 identified molecule (**2**): pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl) (49.34% and 21.81%, respectively), while metabolites: **3** or pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro (16.38%) and hexanedioic acid, mono (2-ethylhexyl) ester (83.62%) were the main constituents of F4 volatile profile (Table S1). Metabolite **2** is an antibacterial volatile substance, previously isolated from *Vibrio parahaemolyticus* (Pandey et al. 2010), and from an endophytic fungus *Penicillium* sp. (Devi

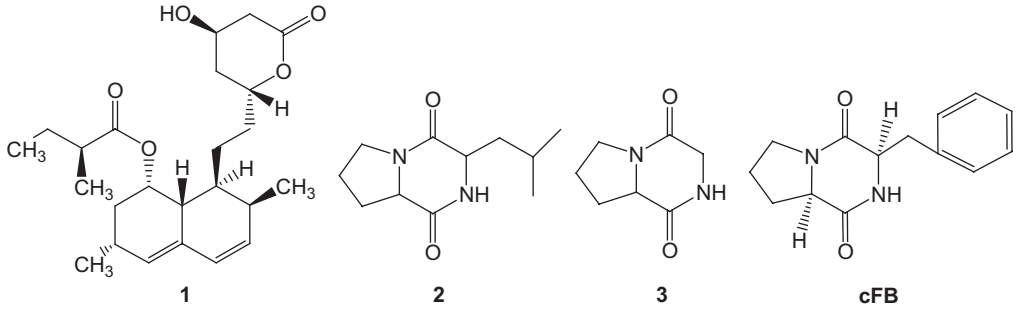

Figure 1. Chemical structures of known fungal molecules **1**–**3** and the autoinducer–signal **cFB.**

& Wahab 2012). Fraction 4 contained **3**, a chain analogue shorter than **2**. The known chemical structures found are similar to bacterial signal (**cFP**) [(3s,9s)-hexahydro-3-(phenyl)-pyrrolo-(1,2-a)-pyrazine-1,4-dione] which is involved in the host–microbe interactions. Its active enantiomer, cyclo (L-Phe-L-Pro), is shown in Figure 1 (Park et al. 2006).

2.7. *Antibacterial and antibiofilm activities of* P. commune *INTA1 metabolites*

2.7.1. *Biofilm quantification after 1 h treatment*

S. aureus ability to form biofilms after 1 h F2 (containing **2**) exposure at 100 $\mu g\,mL^{-1}$ was effectively reduced by 90%. Similar results were observed for F4 (containing **3**), while the odourless crystalline fraction F1 (100 $\mu g\,mL^{-1}$), as well as the lovastain standard, was able to reduce *P. aeruginosa* biofilms by 53% (Figure S4).

It is important to note that the active compound **cFP** was detected from Gram negative bacteria *Vibrio fischeri*, *Vibrio vulnificus*, *Vibrio harveyi* and *P. aeruginosa*, which induced the expression of *V. fischeri lux* reporter system. **cFP** was considered as a signal molecule controlling the expression of important genes for bacteria pathogenicity (Holden et al. 1999). This would explain the stimulant effects of F2-4 (containing analogues of **cFP**) on *P. aeruginosa* biofilms at 1 h post treatment only (Figure S4). On the other hand, *S. aureus* strains utilise small peptides as signal molecules (QS) or bacteriocins (Shpakov 2009). Hence, the mechanism by which the eukaryote natural products contained in F2 and F4 strongly reduce *S. aureus* biofilm architecture could be the disruption of cell to cell communication. However, further studies are needed to isolate pure **2** and **3** and to elucidate their stereochemistry and mode of action as probable peptide-autoinducer antagonists.

2.7.2. *Biofilm formation after 24 h of incubation*

Biofilm formation inhibition after 24 h of F2 exposure at 50 $\mu g\,mL^{-1}$ was 53%, and 10% for a *P. aeruginosa* culture (Figure S5). However, the mixture (M) of F2 at 6 $\mu g\,mL^{-1}$ and oxacillin antibiotic at 3 $\mu g\,mL^{-1}$ was significantly more effective than F2 and oxacillin alone on both Gram positive and negative bacteria with biofilm phenotypes (91% and 38%, respectively). Thus, F2 improved the antibiotic activity.

The lipophyllic properties exhibited by F2, which was completely soluble in organic solvents like AcOEt, and insoluble in water, suggest that their principal targets are cell membranes and their toxicity is caused by loss of chemiosmotic control, as previously reported for similar compounds (Gershenzon & Dudareva 2007). It has recently been proved that the *Piper nigrum* and *Telfairia occidentalis* extracts have synergistic effects with antibiotics. They would probably improve antibiotic penetration in cells via membrane alteration (Noumedem et al. 2013). Thus, the design of synergistic interactions using antibiotics like oxacillin, whose target is the cell wall, is very important. Indeed, bacterial cell walls are unique structures that serve as ideal targets for antimicrobial drugs. Agents that interfere with bacterial cell wall biosynthesis or cell integrity have been used therapeutically with high efficacy and good safety since the 1940s. Because there is no comparable structure in mammalians, bacterial cell wall inhibitors can exhibit high target specificity with side-effect profiles that are not target related, unlike some other antibiotics (Bush 2013).

2.7.3. *Cell growth of* S. aureus *and* P. aeruginosa *strains after 24 h of incubation*

The fungal extract effects on bacterial growth are shown in Figure S6. F2 produced a 37% growth reduction on *P. aeruginosa* cultures (in both concentrations). In general, effects of fungal metabolites on *P. aeruginosa* were more important on cell growth than on biofilm formation.

F1 and lovastatin displayed a weak growth inhibitory activity against Gram positive bacteria (Figure S6) and, in agreement with results shown in Figures S4 and S5, no significant differences were found between both. The coherence of their chemical and biological results was key for determining lovastatin as the main F1 metabolite.

F2 effects ($50\,\mu g\,mL^{-1}$) were more important on *S. aureus* biofilm formation than on bacterial growth, and the moderate cell growth reduction of M (39%) could partially explain the strong effect observed on *S. aureus* biofilm formation (91%, Figure S5). Therefore, another mechanism would be involved, as it was supposed in a previous report on antipathogenic volatiles from *Aspergillus parasiticus* (Cartagena et al. 2014), which does not kill bacteria or stop their growth. Rather, they control bacterial virulence factors such as biofilm, and prevent the development of resistant strains (Otto 2004).

3. Conclusions

The present research provides evidence of the production of lovastatin from *P. commune* INTA1. In fact, the known statin was previously isolated from other fungal strains, and herein lovastatin is reported for the first time as a polyketide-derived natural product from an environmental isolate of *P. commune* mod. In addition, two known pyrrolopyrazine molecules (or cyclic dipeptides) were identified in F2 and F4 by GC-MS. These fractions showed novel antipathogenic effects and F2 significantly improved the antibiotic performance of oxacillin.

Supplementary material

Experimental details relating to this paper are available online, alongside Table S1, and Figures S1–S6 (Results).

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