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Identification of oxylipins with antifungal activity by LC-MS/MS from the supernatant of *Pseudomonas* 42A2

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ABSTRACT

In microorganisms hydroxy fatty acids are produced from the biotransformation of unsaturated fatty acids. Such compounds belong to a class of oxylipins which are reported to perform a variety of biological functions such as anti-inflammatory or cytotoxic activity. These compounds have been found in rice and timothy plants after being infected by specific fungus. When grown in submerged culture with linoleic acid, *Pseudomonas* 42A2 accumulated in the supernatant several hydroxy fatty acids. In this work LC-MS/MS has been used to elucidate the structure of the components form the organic extract: 9-hydroxy-10, 12-octadecadienoic acid; 13-hydroxy-9, 11-octadecadienoic acid; 7, 10-dihydroxy-8E-octadecenoic acid; 9, 10, 13-trihydroxy-11-octadecenoic acid and 9, 12, 13-trihydroxy-10-octadecenoic acid. Antimicrobial activity against several pathogenic fungal strains is presented: MIC (µg/mL) *Verticillium dhaliae*, 32; *Macrophonia phaesolina*, 32; *Arthroderma uncinatum*, 32; *Trycophyton mentagrophytes*, 64.

1. **Introduction**

Although hydroxy-fatty acids are ubiquitous compounds in nature, occurring in animals, plants and fungi. Plants are the main suppliers of hydroxy fatty acids, with castor oil the most common; due to the variety of possible industrial applications of ricinoleic acid, research has been done to find the best method to produce engineered castor oil (Naughton, 1974; Kinney, 2002). In microorganisms hydroxy fatty acids are produced from the biotransformation of unsaturated fatty acids (Culleré et al., 2001; Rodríguez et al., 2001; Kuo et al., 2002). Such compounds belong to a class of oxylipins with antifungal activity which have been found in *Oryza sativa* plants resistant to rice blast disease, and isolated from timothy plants infected by the fungus *Epichloe typhina* (Kato et al., 1988). In addition, these type of compounds are reported to perform a variety of biological functions such as anti-inflammatory activity (Dong et al., 2000) or cytotoxic activity (Yoshiki. et al., 1998). Besides the biological properties, interest in hydroxy fatty acids is increasing due to the industrial applications of these renewable compounds as starting material for resins, emulsifiers, plastics, and lubricants. Hydroxy fatty acids (C18 or C20) are used as thickeners in a new generation of emulsifiers which require polyhydroxy fatty acids to reach new levels of performance (Hagemann and Rothfus, 1991). Various attempts have been made to synthesize such compounds, however the chemical pathway is tedious and produces a low yield (Rama Rao et al., 1987; Li et al., 2009).

Pseudomonas aeruginosa 42A2 has been reported to accumulate hydroxy fatty acids in the cellular compartment in the form of polyhydroxyalkanoates (PHA) and oxylipins such as 10-hydroxy-8E-octadecenoic acid, and 7,10-dihydroxy-8E-octadecenoic acid, in the supernatant when cultivated in oily substrates (Culleré et al., 2001). The aim of this study was the direct structural characterization of the oxylipins accumulated in the culture broth using Liquid Chromatography coupled to tandem Mass Spectroscopy (LC-MS/MS) and the determination of the antifungal activity of these metabolites obtained from cultures of *P. aeruginosa* 42A2 when incubated with commercial linoleic acid as the carbon substrate.

2. **Materials and methods**

2.1 Chemicals

SDS (Carlo Erba Reagenti, Italy) supplied the organic solvents and the HPLCgrade organic solvents. Panreac (Barcelona, Spain) supplied the chemicals, all of ACS quality. ADSA (Barcelona, Spain) and Pronadisa (Barcelona, Spain) supplied the microbiological media. The growth substrate, linoleic acid 60%, was purchased from Sigma-Aldrich Chemie GmbH, Steinheim, Germany.

2.2 Bacterial strain and growth conditions

P. aeruginosa 42A2 (NCIB 40045) was used in this study. After being grown on Tryticase Soy Agar (TSA), cells were cultivated in baffled Erlenmeyer flasks for 24 hours and finally they were inoculated in a bioreactor Biostat® B 2L (Sartorius, Melsungen, Germany). The culture media used was composed of the following salts (g/L): $CaCl_2$ (0,01), $NaNO_3$ (3,5), K_2HPO_4 (2,0), KH_2PO_4 (1,0), KCl (0,1), $MgSO_4 \cdot 7H_2O$ (0,5), $FeSO_4 \cdot 7H_2O$ (0,012) and 0,05 mL/L of a trace elements solution. The trace element solution is as follows: (mg/100 mL): H_3BO_3 (148), $CuSO_4 \cdot 5H_2O$ (196), $MnSO_4 \cdot H_2O$ (154), $Na_2MoO_4 \cdot 2H_2O$ (15), $ZnSO_4 \cdot 7H_2O$ (307). The carbon source added, 2%(v/v) linoleic acid (60%) contained also: 30% of oleic acid and 10% of linolenic acid, palmitic acid and stearic acid. The medium was sterilized separately for 20 min at 121 ºC and 1 bar. The final pH of the medium was adjusted to 7.0. The baffled Erlenmeyer flasks were incubated at 30ºC in an orbital shaker (150 rpm). The medium was inoculated with a 2% v/v Ringer cell suspension (OD 2.0; λ = 540 nm).

The Biostat® B 2L was modified with a ceramic membrane (Audita Unternehmensberatung GmbH) (300 nm pore diameter, 7.5 mm i.d., 13.5 mm o.d.) used as an air sparger; the membrane was attached with an epoxy adhesive (Fast-Metal Minute Adhesive, Weicon GmbH & Co. KG, Münster, Germany) to a stainless steel pipe (5 mm i.d., 5.5 mm o.d.). The process was controlled automatically through the following variable values: pH 7.0, temperature 30 ºC, volumetric air flow 0.20 L min^{-1}, agitation 500 rpm (min 360 rpm), dissolved oxygen 20% (min 10 %) for 54

hours. Data were registered in an external computer connected to the control unit of the bioreactor. The software used was MFCS/win 2.0 (B. Braun Biotech International, Sartorius, Melsungen, Germany).

2.3 Product extraction

At the end of cultivation, after centrifuging, the culture broth was acidified to pH 2 with HCl (37%), followed by an extraction with an equal volume of ethyl acetate (twice). The solvent was evaporated from the combined extracts with a rotary evaporator (Bücchi, Flawil, Switzerland).

2.4 Analysis of products

For LC-MS-MS analysis, a quaternary pump system from Waters model Alliance 2695 was coupled to a PE Sciex API 365 (Applied Biosystems, Foster City, CA, USA) equipped with a turbo ionspray as the ionisation source and a triple quadrupole as the analyser. Data acquisition was carried out by Analyst 1.4 software.

All reported data were acquired in negative mode. The optimal ionisation source working parameters were: nebuliser gas, 10 a.u. curtain gas, 12 a.u temperature 400ºC; turbo ionspray voltage, -3.5 kV; declustering potential, -50 V; focusing potential –200 V; entrance potential, -10 V.

The data acquisition was performed using full scan adquisition resulting in the typical Total Ion Current plot (TIC) and Product Ion Scan (PIS) modes, using as the precursor ion the deprotonated molecular ion [M-H]-; the collision cell offset voltage applied was –15 V with a collision gas pressure of 4 a.u. The scan data were obtained by scanning from *m/z* 80-350 amu.

The chromatographic separation of carbon source and mono- and polyhydroxylated fatty acids was carried out by reverse-phase liquid chromatography using a Tracer Excel 120 C8 column (150mm x 4.6mm, 5µm) (Teknokroma, Barcelona, Spain). Optimum separation was achieved with a gradient elution using: A: acetonitrile (0.1% v/v acetic

acid); B: water (0.1% v/v acetic acid) at a flow rate of 1 mL min^{-1}. Gradient (time, %B): (0, 70); (10, 0); (15, 0); (20, 70); (25, 70). Injection volume was 20 µL.

A known homemade standard mixture of carbon source and mono- and dihydroxylated fatty acids derivatives from oleic acid (Culleré et al., 2001), were used to establish the retention times of the analysed compounds.

2.5 Antimicrobial assay

Bacterial strains tested*:* *Bacillus cereus* var. mycoides CECT 193; *Bacillus subtilis* ATCC 6333; *Bordetella bronchiseptica* ATCC 4617; *Enterococcus hirae* ATCC 10541; *Enterobacter aerogenes* ATCC 13048; *Escherichia coli* ATCC 8739; *Klebsiella pneumoniae* ATCC 145332; *Pseudomonas aeruginosa* ATCC 9027; *Salmonella typhimurium* ATCC 14028; *Serratia marcescens* CECT 274; and *Staphylococcus aureus* ATCC 9341. Fungal strains tested: *Candida albicans* ATCC 10231; *Aspergillus niger* ATCC 16404; *Aspergillus repens* IMI 016114; *Arthroderma uncinatum* ATCC 15082; *Penicillium chrysogenum* ATCC 9480; *Penicillium funiculosum* CECT 2914; *Trycophyton mentagrophites* ATCC 18748; *Verticillium dahliae* 49507/A; and *Macrophomina phaesolina* 48561.

The minimal inhibitory concentration (MIC) of the organic extract was determined against the bacterial and fungal strains by using a broth micro-dilution medium assay (Espinel-Ingroff et al. 2003). Briefly, serial dilutions of the organic extract in 5% DMSO were made in sterile Mueller Hinton Broth (Oxoid, USA) in the case of bacteria and sterile potato dextrose broth (Difco, USA) for fungal strains. A 96-well polypropylene microtiter plate (Costar, Corning Incorporated, Corning, N.Y., USA) was used. Each well, containing 100 µL of the corresponding medium was inoculated with 10µL of test organism to a final concentration of approximately 10^4-10^5 CFU mL^{-1} for bacteria and 10^3-10^4 for spores. Plates were incubated for 18-24 hours at 37 °C for bacteria or 48-96 hours at 25 ºC in the case of fungi. Each treatment was done in triplicate. Parallel controls with DMSO were performed to assess the viability of the assayed strain. The MIC was taken as the lowest concentration at which growth was inhibited after the incubation time. Antifungal activity was assessed on solid media. Potato dextrose agar plates were adjusted to give concentrations ranging from 500 to

1000 ppm. A 5 mm diameter plug of mycelium was placed inverted in the centre of the plate, aseptically. Control plates containing only culture medium were prepared. Samples were made in triplicate. Plates were incubated in the dark at 25 ºC for five days. Colony radii were measured every 24 hours. Inhibition of growth was calculated using the equation:

$$IG\ (\%) = \frac{\text{diameter (mm) in control plates} - \text{diameter (mm) in oxylipin plates}}{\text{diameter (mm) in control plates}}$$

3. **Results and discussion**

The analysis, purification and isolation of fatty acids from bacterial cultures, when a complex carbon source is used, are a difficult task. After organic extraction, current methods used for the analysis of hydroxylated fatty acids include gas chromatography (GC) or gas chromatography coupled to mass spectrometry (GC-MS) analysis; although this allows the compounds to be quantified in minute amounts, it requires the conversion of hydroxy fatty acids into their methyl esters (FAMEs) or trimethyl-silyl ester (TMS) derivatives with the added danger of thermal decomposition or isomerisation (Chen et al., 2007). Liquid chromatography (LC) allows the direct analysis of the products, so this process represents an important advantage. Liquid chromatography coupled to mass spectrometry (LC-MS) and recently the tandem mass spectrometry coupling (LC-MS/MS) has become a powerful technique due to its high selectivity and sensitivity (Masoodi, et al., 2008). LC-MS/MS combines the resolution of LC with the detection specificity of MS/MS, overcoming the limitations of the conventionally approaches and providing a direct and fast methodology to resolve the structure of a pleiad of compounds accumulated in the culture without prior purification or formation of derivatives .

3.1 Analysis of the organic extract

To assess the method used in this study a known mixture of fatty acids was used as standard (Table 1) including: 10-hydroxy-8E-octadecenoic acid (MHOD) and 7,10-dihydroxy-8E-octadecenoic acid (DHOD), produced by bacterial biotransformation as

previously described Culleré et al., 2001; oleic acid (OA) and stearic acid (SA) was tested using LC-MS in negative mode using the acetonitrile/water gradient.

3.2 Identification of hydroxyl fatty acids from batch culture of P. aeruginosa 42A2.

20 μL of the organic extract culture of 54 hours (0.1 mg/mL) dissolved into methanol, was analysed by LC-MS. The Full Scan chromatogram (Fig. 1a) showed a variety of signals corresponding to the formation of different hydroxylated compounds. The Product Ion Scan (PIS) of the *m/z* 329 (related to the $[M-H]^-$ of THOD compounds) showed a sharp peak at RT 4.76 min and small group of peaks between RT 5.28-5.65 min (Fig.1b). The chromatogram showed two the presence of two different isomers: 9,10,13-trihydroxy-11E-octadecenoic acid (9,10,13-11E-THOD) and 9,12,13-trihydroxy-10E-octadecenoic acid (9,12,13-10E-THOD), in the ratio of 3:1 (Fig 1c), differing from that (1:1) found with *Pseudomonas* PR3 (Kim et al., 2002). Mass spectra confirmed those peaks corresponded to THOD compounds (Fig. 2).

These two families of compounds are not easy to separate; therefore a Multiple Reaction Monitoring (MRM) analysis was performed. The MRM acquisition mode is based on the injection of the sample containing the ion to be studied into the first quadrupole (Q1), fragmentation in the collision cell (Q2) and scanning of the product ions in the third quadrupole (Q3). The result is an MS/MS spectrum free of interferences where the most appropriate product ions are selected in the third quadrupole (Q3) to perform relative quantification and confirmation. Considering the fragmentation pattern, it was seen that for the 9,10,13-11E-THOD the characteristic transition was *m/z* 329-127, and for the other family, 9,12,13-10E-THOD, the characteristic transition was *m/z* 329-129. The chromatograms obtained out of the MRM analysis are shown in Figure 1c. Since Kato isolated them from a variety of rice plant suffering from black rot disease, these compounds have gained attention (Kato et al., 1985) as part of the oxylipins involved in the defensive mechanism of plants, however few references are found in the literature about the bacterial transformation of polyunsaturated fatty acids into polyhydroxy fatty acids. Hou reported the synthesis of 12,13,17-trihydroxy-9Z-octadecenoic acid by *Clavibacter,* a Gram-negative bacteria (Hou, 1996) or 7,10,12 trihydroxy-8E-octadecenoic acid -in minor amounts (mg/L)

produced by strain PR3 of *Pseudomonas* from ricinoleic acid (Kuo. et al., 2001) or from linoleic acid, 9,10,13-trihydry-11E-octdecenoic acid and 9,12,13-trihydroxy-10E-octadecenoic acid in equimolecular amount (Kim et al., 2000); biotransformation was positively affected by the presence of Fe^{2+} or Cu^{2+}. Finally, several strains of *Bacillus megaterium* converted linoleic acid into 12,13,17-trihydroxy-9Z-octadecenoic acid and 12,13,16-trihydroxy-9Z-octadecenoic acid (Hou, 2005).

3.3 Identification of hydroxyl fatty acids from feed-batch culture of P. aeruginosa 42A2

Changing the feeding strategy of the culture, by supplying 20 g/L of carbon substrate, after 6.5 hours of incubation, the transformation products appeared sequentially. After only two hours of incubation, the monohydroxylated compound was already detected approaching the maximum (4 g/L) at 4 hours; the dihydroxylated fatty acids appeared after 6 hours of incubation, and increased up to 2 g/L; the trihydroxylated compounds, appeared late, after 28 hours of incubation, reaching their maximum of 0.5 g/L at 30 hours. After 70 hours of cultivation, cells were collected and supernatants acidified for organic extraction and analysed by LC-MS. The TIC chromatogram showed the formation of additional hydroxylated compounds (Fig. 3).

Two new hydroxylated fatty acid were detected 9-hydroxy-10,12-octadecedienoic acid and 13-hydroxy-9,11-octadecadienoic acid. These compounds might be due to the lipoxygenase of *Pseudomonas* 42A2, (Vidal-Mas et al., 2005), since it was reported that the lipoxygenase of the fungus *Gaeumannomyces graminis* (Garscha et al., 2007), the cyanobacteria *Nostoc punctiforme* (Koeduka et al., 2007) or *Anabaena* (Zheng et al., 2008) converts linoleic acid into these compounds. Finally, the 10-hydroxy-8E octadecenoic acid detected is due to the conversion of oleic acid (Culleré et al., 2001; Kuo et al., 2008). Other monohydroxy fatty acids reported in the literature were 10-hydroxy-12Z-octadecenoic acid by strains of *Nocardia*, *Flavobacterium*, *Enterococcus, Lactobacillus and Stenotrophomonas* (Yu et al., 2008) and 3-hydroxy-fatty acids accumulated by *Lactobacillus plantarum* (Sjörgren et al., 2003).

In this way up to six different acids were easily identified, without having to produce and analyze derivatives, from the supernatant of the bacterial culture. To our knowledge, no reports exist of the identification of 9-hydroxy-10,12-octadecadienoic acid, and 13-hydroxy-9,11-octadecadienoic acid, from cultures of *Pseudomonas*.

3.4 Biological properties

Higher plants convert C18 unsaturated fatty acids into mono-, di- or trihydroxy fatty acids. Many of these hydroxy fatty acids belong to the defence mechanism and form part of the oxylipins (Bleé 1995; Prost et al., 2009). First, Kato, et al. extracted from an infected rice plant suffering from blast rot disease, 9S,12S,13S-trihydroxyoctadeca-10E,15-dienoic acid and 9S,12S,13S-trihydroxyoctadeca-10E-enoic acid, which act against the rice blast disease, the fungal infection produced by *Pyricularia oryzae* (Kato et al., 1985). The 9S,12S,13S-trihydroxyoctadeca-10E-enoic acid was also isolated from *Colocassia antiquorum* and showed anti-black-rot fungal activity (Masui and Kojima, 1989). It was found that 9,10,13-11E-THOD and 9,12,13-10E-THOD reduced infection up to 42%, when applied *in vivo*, of the mildew fungus in barley, *Blumeria graminis* (Cowley and Walters, 2005). 10-hydroxy-8-octadecenoic and 10-hydroxy-8,12-octadecenoic acid were found in the stromata of the timothy plant after being infected with *Epichloe thyphina* (Koshino et al., 1987). Although the mechanism of action of oxylipins is not yet understood, Prost et al. have suggested that the inhibition of growth may be due the chemical or physical properties of the oxylipins rather than the to interactions with specific cellular targets t (Prost et al., 2009).

As presented above, the organic extract from the culture of *P. aeruginosa* 42A2 on linoleic acid (60%) contains several oxylipins similar to those considered signalling molecules in the plant kingdom, and may be consider as an unique product for application. Under this premise, the antimicrobial properties of several pathogenic fungal strains were assayed (Table 3): high activity (32 µg/mL) was found against *P. funiculosum*, the agent causing white streaks in the kernel pericarp and human allergen; similar MIC values (from 25-100 µg/mL) were reported with the oxylipins produced by *Lactobacillus* (Sjörgren et al., 2003). The MIC found for the queratinolitic fungi *Artrhoderma uncinatum* and the dermatophitosis etiological agent *Trycophiton*

mentagrophites was 32 µg/mL and 64 µg/mL respectively. Complete growth inhibition was found at 140 µg/mL against the allergenic strains, *Aspergillus niger*, *Aspergillus repens* and *Penicillium chrisogenum*

Growth inhibitions found in solid cultures were assayed: *Fusarium oxysporum,* the causal agent of root and crown rot of tomato, presented 45% of growth inhibition at 50 ppm; at this concentration the 10-hydroxy-8E-octadecenoic acid (MHOD) and 7,10 dihydroxy-8-octadecenoic acid produced 54.5 and 56.5% growth inhibition respectively. The 3-(R)-hydroxy derivatives produced by *Lactobacillus plantarum* were found to be active against strains of *Penicillium*, *Aspergillus* and *Kluyveromyces* and the yeast strains, *Rhodotorula* and *Pichia* (Sjörgren et al., 2003). The whole extract from *Pseudomonas* 42A2 inhibited 60% of the growth of *Colleotrichum gloespoioides* at 500 ppm, compared with the 19 or 28% of inhibition found when pure MHOD or DHOD was tested. Higher reduction (90%) against *Drechlera teres* was found at 500 ppm, whereas 750 ppm was needed to reduce the micelar growth of *P. chrysogenum* (60%) Finally, the MIC presented in Table 3 was found against the Gram-positive strains, whereas no inhibition could be observed in the Gram-negative strains assayed or against *Candida*.

4. **Conclusions**

In the course of the biosynthesis of hydroxyl fatty acids produced by *Pseudomonas* from a complex substrate. LC coupled to MS/MS was found to be a versatile, rapid and sensitive method for the study of the time course of biosynthesis of hydroxyl fatty acids by *Pseudomonas* from a complex substrate. Using this method we identified (up to seven) mono-, di- and trihydroxy fatty acids without prior production of derivatives some being detected for the first time: 9-hydroxy-10,12-octadecedienoic acid, and 13-hydroxy-9,11-octadecedienoic. Without further separation/purifiaction, the oxylipins accumulated in the supernatant may be used against bacterial and phytopathogenic fungal strains.

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