http://dx.doi.org/10.5935/0100-4042.20140045 *Quim. Nova,* Vol. 37, No. 2, 260-267, 2014

Artigo

BIOMOLECULES PRODUCED IN LIQUID-STATE FERMENTATION BY A MARINE-DERIVED FUNGUS, *Penicillium roqueforti*

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Recebido em 04/06/2013; aceito em 20/09/2013; publicado na web em 01/11/2013

Screening of biomass of a new marine-derived strain of *Penicillium roqueforti*, as produced by liquid-state fermentation, led to the identification of several volatile organic compounds active in the fatty acid pathway as well as fragments produced by their catabolism, terpenoids, and metabolites from the shikimic acid pathway. In addition, five non-volatile organic compounds, triolein, ergosterol peroxide, 9(11)-dehydroergosterol peroxide, 4-hydroxybenzaldehyde, and D-mannitol, were isolated and identified by spectroscopy. The results showed that this fungal strain did not produce any mycotoxin in the culture conditions applied, and thus is useful for industrial applications, where high value-added biomolecules are generated.

Keywords: *Penicillium roqueforti*; chemical characterization; high value-added biomolecules.

INTRODUCTION

The filamentous fungus *Penicillium roqueforti* is well-known for its use in biotechnological applications and has been extensively used in the dairy industry to add flavor and veining to internally mould--ripened blue cheeses.[1] It is a common contaminant mould found in silages, food, and feed, and its ability to produce a large number of biologically active extrolites, including various toxins, has attracted the interest of many researchers.[2]

Although it is described as a terrestrial fungus, some studies have shown that *P. roqueforti* strains have high salt tolerance,[3] with spore germination inhibited only at sodium chloride concentrations of over 100 g L^{-1}.[4] This extraordinary tolerance to salt, along with its tolerance to high osmotic pressure, indicates that this hyphomycete adapts to life as a marine facultative fungus.[5] Note that the average seawater salinity is 35 g L^{-1}.

Moreover, under certain culture conditions, this fungus has shown its pronounced capacity to biosynthesize secondary metabolites,[6] some of which have antiparasitic[7] and bacteriostatic properties.[8] Its proteolytic enzymes allow it to be applied in various processes, such as removal of scales of some fish, in the fishing industry[9] and in biotechnology for the production of high-quality fat from food waste.[10]

With regard to the *P. roqueforti* chemical composition, it is worth noting the extensive research performed on its fatty acid profile and lipid metabolism.[11] Chromatographic studies on the total lipid fraction of *P. roqueforti* have shown the presence of palmitic, stearic, oleic, and linoleic acids sterified in the form of acyl-glycerides and free fatty acids, whereas the more polar lipids are composed of phospholipids and glycolipids,[12] and the free steroids have an ergosterol skeleton.[13] Further data are available on the biogenesis of unsaturated fatty acids depending on the phases of growth.[14]

However, the applicability of this fungi in foodstuffs became limited after the discovery that these organisms are also capable of producing dangerous secondary metabolites.[15,16] These toxic strains can be identified using GC–MS in line with the chemical tracers proposed by Demyttenaere *et al*.,[17] Jelen,[18] and Calvert *et al.*[19]

P. roqueforti produces several mycotoxins, such as PR-toxin, roquefortine C, mycophenolic acid, patulin, and penicillic acid,[20] some of which are known to be generally unstable or incapable of causing serious damage at low concentrations.[21] Although they can occur under natural conditions in feed, both roquefortine C and mycophenolic acid are considered to be low-toxicity mycotoxins with less significance.[20-22] PR-toxin is the most significant because it is reported to cause damage to the liver and kidney in rats[16] and is also potentially carcinogenic.[23] However, it should be noted that fungal metabolite production depends on several variables such as the isolate,[24] growth medium, and environmental factors.[25]

Thus, the present study examined the metabolites biosynthesized by the fungus *P. roqueforti* that was isolated from marine biota and grown in marine culture media. Detailed screening of mycelia was performed and volatile and non-volatile organic compounds were identified using GC–MS and NMR spectroscopy. It is anticipated that the chemo-specific information may offer crucial information for improving its biotechnological applications.

EXPERIMENTAL

Isolation and identification of the fungus

The halotolerant fungal strain was obtained from marine water collected at the intertidal zone of the "La Laja" beach, Gran Canaria, the Canary Islands, Spain. Water samples were randomly collected in sterile flasks (24 units; 125 mL each) at 0.2-0.5 m depth in accordance with the procedures proposed by Seymour and Fuller.[26] Each water sample (0.5 mL, undiluted) was spread on Petri dishes, and the plates were incubated at 26 °C (±2 °C) for 21 days. The isolation process and strain purification were performed on Petri dishes using a modified KMV solid medium containing 1 g yeast extract, 1 g hydrolyzed gelatin, 1 g peptone, 5 g glucose, and 12 g bacteriological agar in 1 L filtered seawater (salinity, 35 ppt). The fungus was identified as *P. roqueforti* using morphological criteria defined by CABI Bioscience, Surrey, UK (see supplementary material), and a voucher specimen was deposited at the laboratory for future references under the accession number PA 002.

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Liquid-state fermentation

After the purification of colonies, the mycelial spores were scraped and transferred to Erlenmeyer flasks (2 L capacity) containing the sterile modified KMV broth, which was autoclaved at 121 °C for 20 min. The modified KMV broth contained 1 g yeast extract, 1 g hydrolyzed gelatin, 1 g peptone, and 5 g glucose in 1 L filtered seawater.

Fungal biomass production was performed in liquid-state fermentation in static polypropylene boxes (83 × 46 × 18 cm) that were previously sterilized with sodium hypochlorite and steamed for 5 min. Once the steam was condensed, water was drained out of the boxes. The spore solution was then homogenized for 10 min in a magnetic mixer using a bar stir and transferred directly to each box (20 units with 1.2 L of culture broth/unit). After 10–12 days of incubation at room temperature (22–25 °C), the supernatant mycelia were separated by filtration and dried by IR radiation.

Apparatus and analytical methods

Normal-phase chromatography was performed on silica gel (Scharlau) using a 0.06-0.2 mm particle sized adsorbent and a 0.04-0.06 mm particle sized stationary phase. Chromatography was performed at medium (Büchi Chromatography System) or low pressure using Fluid Metering Inc. motors connected in series with an Ace Glass Inc. column. Reverse-phase chromatography was performed on a LiChroprep RP-18 (40-63 μm particle size; Merck) column connected to a low-pressure chromatography system also based on the Fluid Metering Inc. apparatus.

Size-exclusion chromatography was performed on lipophilic Sephadex® LH-20 (Sigma). The column was eluted first with anhydrous methanol (2 h) and then with a mixture of CH_2Cl_2/CH_3OH (50:50, 2 h). The extracts were applied at the top of the column and eluted with CH_2Cl_2/CH_3OH (50:50) at a rate of 1.0 mL min^{-1}.

Normal-phase TLC was performed on silica gel plates (0.25 mm diameter; Tracer Analitica) using a combination of *n*-hexane, ethyl acetate, chloroform, and methanol as the eluent, in the proportions specified for each. Reverse-phase TLC was performed on RP-18F_{254} plates (0.25 mm; Merck) using CH_3CN/CH_3OH/H_2O (80:18:2) as the other mobile phase. In all cases, spots were revealed by spraying with oleum [sulfuric acid (4%) + acetic acid (80%) + water (16%)] and heating at 120 °C for 20 min.

Normal-phase semipreparative HPLC was performed using an Alltech Econosphere silica column (10 μm particle size, 250 × 4.6 mm, and 100 Å pore size), and reverse-phase semipreparative HPLC was performed on a Waters ODS column (10 μm particle size, 250 × 4.6 mm, and 100 Å pore size). Both these processes were conducted using a semipreparative HPLC apparatus coupled with a Spectra-physics P100 isocratic pump and in line with a Hewlett Packard 1050 UV-vis variable wavelength detector working at room temperature (26 °C). Analytical chromatography was performed using a Shimadzu HPLC system with an LC-9A pump connected to a UV SPD-6AV detector (254 nm). The conditions used for the normal-phase column were combinations of *n*-hexane and ethyl acetate as the eluent; in case of the size-exclusion chromatography column (Shodex OH Pak SB 806 HQ), a mixture of water and 0.05% sodium azide was used as the eluent. An eluent flow rate of 1.0 mL min^{-1} was used for all analyses.

Infrared spectra were recorded on a Shimadzu FTIR-8400S spectrophotometer, with chloroform (Merck) as a solvent for spectroscopy. The samples were sandwiched between two sodium chloride plates, and the spectrum was calibrated against the 1603 cm^{-1} band of polystyrene.

^1H, ^{13}C, and 2D-NMR experiments were recorded at 250 or 300 MHz on AC or AMX Bruker apparatus, respectively. A Varian UNITY INOVA 400 MHz NMR spectrometer was used for high-resolution analysis. Tetramethylsilane was used as an internal standard for ^1H, and deuterated chloroform (δ 77.00) or deuterated methanol (δ 49.00) was used for the calibration of 13-carbon NMR spectra.

Electrospray ionization mass spectrometry was performed either at low or high resolution with a common electron impact mass spectrometer (IE) or by fast atom bombardment (FAB). Positive mode was performed on a FAB-MS at 70 eV with a FISONS VG Micromass Autospec apparatus, with NBA (3-nitrobenzyl alcohol) as the matrix. Melting points were established using a Gallenkamp apparatus and were left uncorrected.

Gas chromatography–mass spectrometry (GC–MS) was performed on a chromatograph model Varian CP3800 with an ion-trap mass spectrometer model Saturn 2000 and under the following conditions: CP-Sil 8 low bleed/MS capillary column. The injector temperature was kept isothermal at 270 °C, initial split conditions *on*, and 0.01 min *off* and 5 min *on*, with a split ratio of 1:50; the oven was set at 50 °C for 5 min and then ramped at 15 °C min^{-1} to 250 °C and held for 10 min (for a total run time of 28.33 min for each sample) with a flux of 1 mL min^{-1}, using the mass detector in the EI mode (20–400 m/*z*).

Compounds lacking reference standard were quantified using the response factor for alkanes (Dr. Ehrenstorfer GmbH Alkanes-Mix 10), fatty acid methyl esters (SupelcoTM 37 Component FAME Mix), 1-alkenes (Fluka Chemika), and 1-alkanols (Fluka Chemika). The remaining compounds were assigned by structural analogy to the above. Thus, tetradecanoic acid 1-(hydroxymethyl)-1,2-ethanediyl ester was assigned to the factor obtained experimentally for hexadecanoic acid methyl ester (764.117 × 10^{-12} mg K $counts^{-1}$). This same factor was used for other FAME such as tetra-unsaturated 6,9,12,15-docosatetraenoic acid methyl ester.

RESULTS AND DISCUSSION

Chemical analysis

Each box of culture broth (1.2 L) yielded 38.71–49.67 g of fresh mycelia, corresponding to 3.75–5.42 g of dry matter. The total wet mass of mycelia resulting from the sum of yields was 888.34 g, which produced 89.9 g of dry matter after IR desiccation. The crude extract was obtained by maceration in CH_2Cl_2 (×3, 24 h) and CH_3OH (×3, 24 h) at room temperature. After filtration, evaporation, and vacuum desiccation, 14.8 g of brown oil was obtained. The whole extract was fractionated by polarity in a liquid–liquid extraction system, according to a modified version of the Kupchan method.[27] The process flow diagram can be found in supplementary material (Figure 2S). Each fraction was screened using chromatography (column chromatography, size-exclusion chromatography, and thin-layer chromatography) and analyzed using GC–MS (for volatile compounds) or spectroscopy (NMR, MS, and IR), allowing the identification of the following categories of compounds:

Volatile organic compounds

Volatile organic compounds, i.e., compounds that volatilize in gas chromatograph injector at temperatures of 270 °C, were identified by GC–MS (Table 1) and classified by structural criteria (Figures 1–3) as follows: *n*-alkanes (**1**); 1-alkenes (**2**); 1-alkanols (**3**); 2-alkyl-1-alkanols (**4**); saturated (**5**) and unsaturated (**7**) free fatty acids; fatty acid amides (**10**); saturated (**6**) and unsaturated (**8, 11-14**) fatty acid methyl and ethyl esters; unsaturated triglycerides (**15**) and diglycerides (**16**); unsaturated

Table 1. GC–MS of volatile organic compounds produced by *P. roqueforti* mycelia

N°	R_t (min) (mean ± SD)	Compound (structure[1]); concentration (mg × kg^{-1})
1	12.075 ± n.d.	Dodecane (**1**, n = 9); 0.004
2	12.437 ± n.d.	1-Dodecanol (**3**, n = 10); 0.002
3	12.524 ± 0.007	2-Butyl-1-octanol (**4**, n = 3, m = 3); 4.3
4	13.235 ± n.d.	Tetradecane (**1**, n = 11); 0.07
5	13.252 ± n.d.	2-Hexyl-1-octanol (**4**, n = 3, m = 5); 0.6
6	13.454 ± n.d.	Benzaldehyde, 4-hydroxy (**26**); 1.1
7	13.684 ± 0.004	Non-anionic acid, 9-oxo-, methyl ester (**21**); 39.7
8	13.972 ± n.d.	1-Pentadecene (**2**, n = 12); 1.1
9	14.005 ± n.d.	Geranyl isovalerate (**23**); 114.4
10	14.471 ± n.d.	1-Dodecanol, 3,7,11-trimethyl (**24**); 3.78
11	14.751 ± n.d.	Tetradecanoic acid, 1-(hydroxymethyl)-1,2-ethanediyl ester (**16**); 0.6
12	14.771 ± 0.005	Tetradecanoic acid (**5**; n = 12); 0.01
13	14.843 ± 0.002	1-Hexadecene (**2**; n = 13); 10.7
14	14.857 ± 0.010	1-Tridecanol (**3**; n = 11); 3.2
15	15.642 ± 0.000	Heptadecane (**1**, n = 14); 0.3
16	15.655 ± 0.015	2-Hexyl-1-decanol (**4**, n = 5; m = 5); 4.1
17	15.890 ± 0.008	Tetradecanoic acid, methyl ester (**6**, n = 12); 1.8
18	15.906 ± n.d.	Pentadecanoic acid (**5**, n = 13); 2.5
19	16.338 ± 0.010	1-Hexadecanol (**3**, n = 14); 3.6
20	16.348 ± 0.022	1-Eicosanol (**3**, n = 18); 20.0
21	16.418 ± n.d.	Octadecane (**1**, n = 15); 2.2
22	16.501 ± n.d.	9-Hexadecenoic acid, tetradecyl ester, (Z)- (**20**, n = 5); 1.6
23	16.538 ± n.d.	9-Hexadecenoic acid(**7**, n = 5, m = 7); 11.2
24	16.604 ± 0.020	Pentadecanoic acid, methyl ester (**6**, n = 13); 32.2
25	16.711 ± n.d.	2-Pentadecanone, 6,10,14-trimethyl- (**25**); 15.9
26	17.160 ± 0.015	9-Hexadecenoic acid, methyl ester, (Z)- (**8**, n = 5, m = 7); 382.7
27	17.273 ± 0.006	Pentadecanoic acid, 14-methyl-, methyl ester (**9**, n = 11); 166.9
28	17.289 ± 0.019	Hexadecanoic acid, methyl ester (**6**, n = 14); 1884.1
29	17.928 ± 0.020	Heptadecanoic acid, methyl ester (**6**, n = 15); 9.7
30	18.352 ± n.d.	Tridecanoic acid, 13-formyl-, ethyl ester (**22**); 1.1
31	18.360 ± 0.008	9-Octadecenamide (**10**); 9.6
32	18.385 ± 0.004	11-Octadecenoic acid, methyl ester (**8**, n = 5, m =9); 291.2
33	18.389 ± n.d.	Triolein (**15**); 2.9
34	18.394 ± 0.009	8,11-Octadecadienoic acid, methyl ester (**11**, n = 5, m = 6); 100.8
35	18.550 ± 0.028	Octadecanoic acid, methyl ester (**6**, n = 16); 60.7
36	18.558 ± n.d.	Heptadecanoic acid, 16-methyl-, methyl ester (**9**, n = 13); 11.6
37	18.811 ± n.d.	9,12-Octadecadienoic acid (Z,Z)-, 2-hydroxy-1-(hydroxymethyl) ethyl ester (**17**); 0.7
38	18.962 ± n.d.	Eicosanoic acid (**5**, n = 18); 30.9
39	19.542 ± n.d.	7,10,13-Eicosatrienoic acid, methyl ester (**13**); 140.2
40	19.748 ± 0.005	Tricosane (**1**, n = 20); 5.9
41	19.793 ± 0.024	9-Octadecenoic acid (Z)-, 9-octadecenyl ester, (Z)- (**19**); 11.1
42	19.814 ± 0.004	13-Docosenoic (*erucic*) acid, (Z)- (**8**, n = 7, m = 11); 10.3
43	19.817 ± 0.001	9-Octadecenoic (*oleic*) acid (Z)-, tetradecyl ester (**20**, n = 7); 65.0
44	20.205 ± n.d.	6,9,12,15-Docosatetraenoic acid, methyl ester(**14**); 86.0
45	21.454 ± n.d.	9,12-Octadecadienoic (*linoleic*) acid (Z,Z)-, ethyl ester (**12**); 68.7
46	21.820 ± n.d.	Pentacosane (**1**, n =22); 31.8
47	22.143 ± n.d.	9,12-Octadecadienoic (*linoleic*) acid (Z,Z)-, 2,3-dihydroxypropyl ester (**18**); 48.9
48	22.244 ± n.d.	Docosanoic acid, methyl ester (**6**, n = 20); 5.1
49	22.486 ± n.d.	1-Docosanol (**3**, n = 20); 25.4
50	25.823 ± n.d.	Tetracosanoic acid, methyl ester (**6**, n = 22); 12.7

[1]"Structure" refers to the compound number in figures; R_t = Retention time; n.d. = no date.

monoglycerides (**17**); wax esters (**19** and **20**); lipid catabolites (**21** and **22**); mono-, sesqui-, and straight-chain terpenes (**23-25**); and an aromatic hydrocarbon from the shikimic acid route (**26**).

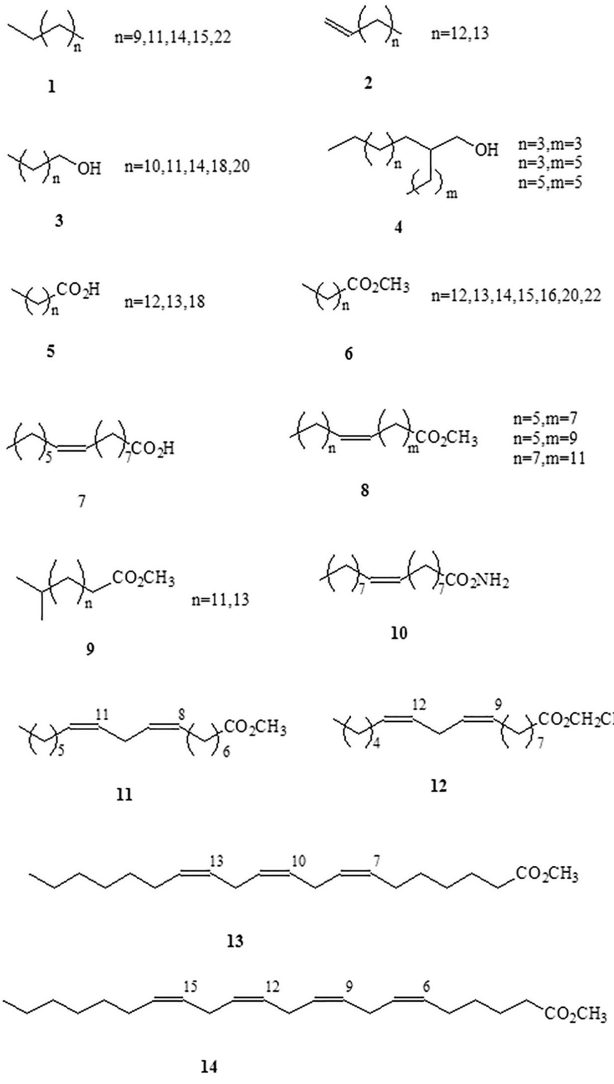

Figure 1. *Volatile lipid compounds identified in Penicillium roqueforti*

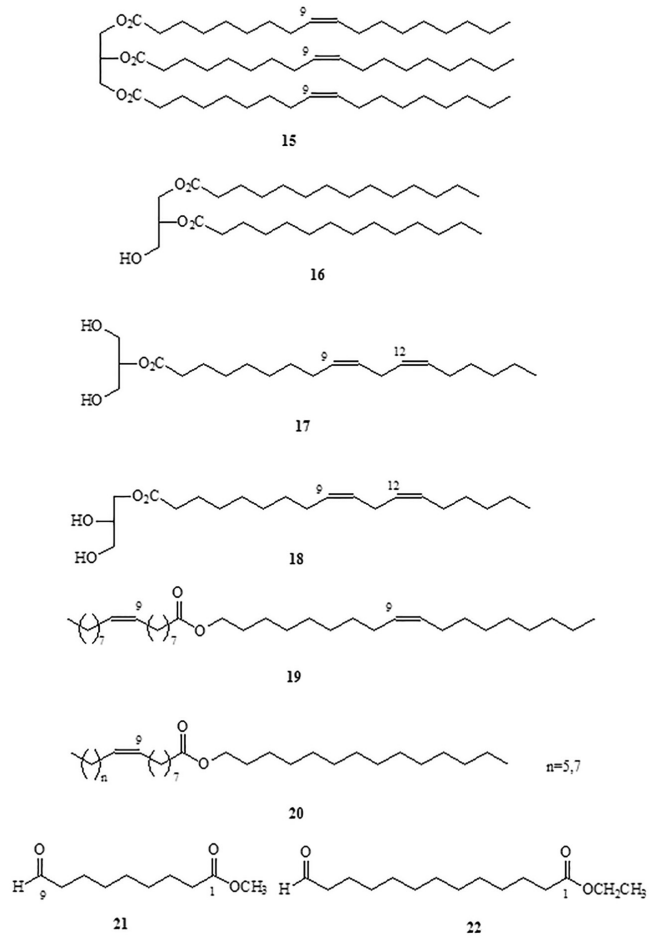

Figure 2. *Volatile lipid compounds identified in Penicillium roqueforti*

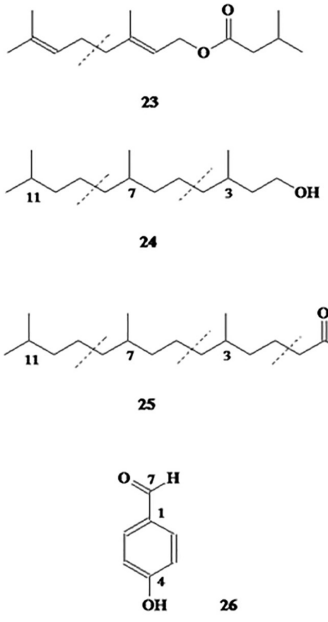

Figure 3. *Volatile terpenoid/shikimate compounds identified in Penicillium roqueforti*

Occurrence of methyl-branched fatty acids

Methyl-branched fatty acids are present as minor lipid components in various living organisms and as major components of lipids in various bacteria.[28] It has been ascertained that they are formed by the selective incorporation of methylmalonyl-CoA, catalyzed by the fatty acid synthetase enzyme.[29]

This biogenetic pathway is a characteristic of bacteria that produce relatively high concentrations of these *iso*-methyl-branched fatty acids, which are therefore accepted as molecular markers of organic matter produced by these organisms.[30] Therefore, identification of methyl ester in 14-methyl-pentadecanoic acid (**9**, n = 11) and 16-methyl-heptadecanoic acid (**9**, n = 13), the two *iso*-methyl--branched fatty acids derivatives, is an indirect evidence of the presence of bacteria associated with this fungus. Apart from the GC–MS fingerprint, the *iso*-methyl-substitution proposed in **9** (n = 11 and 13) was confirmed by the relatively intense fragment ion peak at M^+-43 (m/*z* = 227 and 255, respectively) observed using GC–MS, together with a decrease in the intensity of the M^+-29 (m/*z* = 241 and 269, respectively) fragment.[31]

Non-volatile organic compounds

Non-volatile compounds obtained from *P. roqueforti* mycelia were fractionated and measured by gravimetric analysis and integration

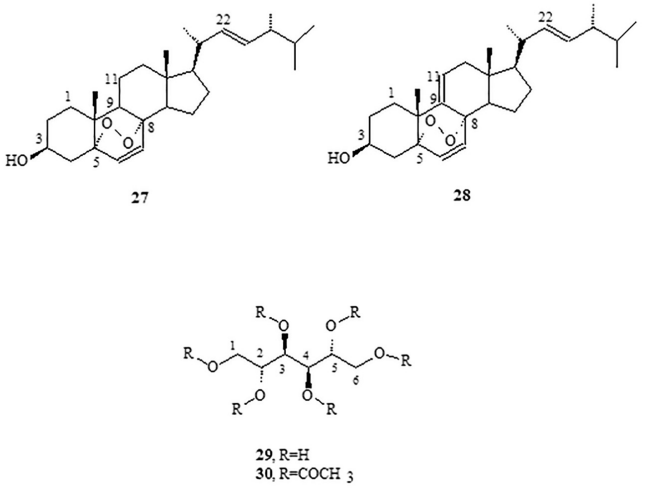

Figure 4. *Non-volatile compounds identified in Penicillium roqueforti*

of the ^{1}H-NMR spectrum and were classified as high-molecular weight alkanes (1.29%), waxes (0.37%), unsaturated steroidal waxes (1.11%), other waxes (2.73%), unsaturated triglycerides [triolein (**15**) and other, 2.56%], ergosterol peroxide (**27**, 12.63%), 9(11)-dehydro-ergosterol peroxide (DHEP; **28**, 2.15%), 4-hydroxybenzaldehyde (**26**, 0.92%), unidentified phospho- and glycolipids (0.31%), mannitol (**29**, 5.0%), and unidentified polyhydroxy compounds (70.93%). Note that some of these described compounds were also detected as volatile components in the previous section.

The major substances, triolein, ergosterol peroxide (EP), 9(11)-DHEP, and mannitol, were the only compounds to be purified and characterized in these non-volatile lipid compounds (Figure 4). Among monocyclic aromatic compounds from the shikimic acid route, 4-hydroxybenzaldehyde could be purified and characterized (Figure 3).

Triolein (**15**) was obtained from one of the less polar fractions of the mycelia extract as yellowish oil and was identified using IR, ^{1}H-NMR, ^{13}C-NMR, ^{1}H-^{1}H-(COSY, TOCSY, NOESY), DEPT_135, HSQC, HMBC, and mass spectroscopic data.

Although several factors may affect either the fatty acid composition or the percentage of total lipids found in fungi,[32] fatty acid analysis of the cultured *P. roqueforti* indicates a limited capacity of lipid accumulation in this strain. However, the characteristic oleic acid as a major constituent of the unsaturated fatty acid fraction (0.4% DW of mycelia) offers functional benefits of oxidative stability and nutritional attributes to this fungus.

Another biomolecule that was isolated in its purest form was 5α, 8α-epidioxyergosta-6,22-dien-3β-ol (**27**) usually denominated ergosterol peroxide (EP). The product, which crystallizes from methanol, has a melting point of 178–180 °C and was identified using IR, ^{1}H-NMR, ^{13}C-NMR, ^{1}H-^{1}H-(COSY, TOCSY, NOESY), DEPT_135, HSQC, HMBC, and mass spectroscopic data (Table 2).

Previous studies have offered contradictory explanations for the origin of sterol peroxides that may be formed by the photo-oxygenation of sterols with double conjugated bonds at C-5/C-6 carbons and at C-7/C-8 carbons.[33] Thus, EP in extracts from fungi was regarded as an artifact rather than as a natural product, which was produced by the photo-oxygenation of ergosterol during the experimental procedures or was possibly sensitized by fungal pigments during mycelia growth.[34]

Although there are no references to the existence of sterol peroxides as biological membrane constituents, enzymatic and photo-oxidative conversions of ergosterol into EP were observed *in vivo* in *Penicillium* sp. and *Gibberella* sp.[22] Similarly, Sheikh and Djerassi[35] working with a sterol mixture from the marine sponge *Tethya aurantia* suggested that EP was formed by biological processes.

Table 2. Ergosterol peroxide (**27**) spectra (^{13}C-NMR and ^{1}H-NMR, $CDCl_3$, 400 MHz)

C	δ (^{13}C)	δ (^{1}H)
1	34.683	1.0-2.2 (20 H, m)
2	30.106	
3	66.465	3.93 (m, 1H)
4	28.630	
5	82.135	
6	135.390	6.20 (1H, d, J = 8.40 Hz), syst. AB
7	130.733	6.46 (1H, d, J = 8.40 Hz), syst. AB
8	79.406	
9	51.081	
10	36.956	
11	20.866	
12	39.335	
13	44.550	
14	51.671	
15	23.389	
16	29.686	
17	56.195	
18	12.859	0.771 (3H, s)
19	18.163	0.839 (3H, s)
20	39.712	
21	19.624	0.954 (3H, d, J = 6.4 Hz)
22	135.183	5.08 (1H, m)
23	132.295	5.12 (1H, m)
24	42.760	
25	33.053	
26	19.932	0.772 (3H, d, J = 6.8 Hz)
27	20.617	0.788 (3H, d, J = 6.4 Hz)
28	17.547	0.863 (3H, d, J = 6.8 Hz)

EP is also a major antitumor sterol produced by edible or medicinal mushrooms.[36-38] This compound can be either extracted from another filamentous fungal species such as *Paecilomyces variotii*,[39] *P. tenuipes*,[40] and *P. herquei*[41] or synthesized from ergosterol by photosensitized oxygenation with eosin.[42]

The 9(11)-dehydro derivative (**28**) was not isolated in its purest form, and it was found with the aforementioned EP (**27**) in the form of a white solid, with a melting point (Mp) of 171 °C–176 °C $[\alpha]_D^{20}$ = –9.7 ($CHCl_3$, c 1.2), and was identified using IR, ^{1}H-NMR, ^{13}C-NMR, ^{1}H-^{1}H-(COSY, TOCSY, NOESY), DEPT_135, HSQC, HMBC, and mass spectroscopic data. The ^{1}H-NMR spectrum showed two superimposed AB systems centered at δ 6.41 and 6.48, respectively, with J = 8.7 Hz for both. After consulting the literature, this material was recognized as a mixture of EP and its 9(11)-dehydro derivative.[43] The quantitative ratio of these products in the mixture was obtained by integrating the olefinic protons in the AB system NMR spectrum (400 MHz), resulting in a mix of EP (79%) and 9(11)-DHEP (21%), structures **27** and **28**, respectively.

This quantitative relation was confirmed using the optical rotation method. A graph was then drawn using the experimental values of optical rotation of the mixture versus the percentage of EP. The graph of the optical rotation data provided by Fisch *et al.*[42] and Mediavilla[43] (Table 1S in the supplementary material) showed a linear relationship between the value of $[\alpha]_D$ and the percentage of EP, where

$$\% \text{ EP} = 70 - 0.91 \times [\alpha]_D$$

By introducing the value $[\alpha]_D^{20} = -9.7$ into this equation, the optical rotation obtained for this material was 78.8% for EP, which was consistent with the 79.0% optical rotation obtained by integrating the ^{1}H-NMR spectrum.

Finally, it should be noted that in the ^{13}C-NMR spectrum of this mixture, six small signs of olefinic carbons can be detected from the DHEP, with the signals assigned relevant for C-9 carbon at δ 130.970 (δ 51.081 in EP) and C-11 carbon at δ 125.00 (δ 20.866 in EP). Moreover, the ^{1}H-NMR spectrum of this mixture gives a signal at δ 5.48 (0.21 H, m) that has been assigned to vinyl proton on C-11 carbon.

Another non-volatile compound, 4-hydroxybenzaldehyde (**26**), was obtained pure by crystallization of methanol (Mp = 121–122 °C). Its structure was elucidated from its spectroscopic data, mainly NMR spectroscopy (Table 3).

Table 3. ^{13}C and ^{1}H-NMR spectra from 4-hydroxybenzaldehyde (CD_3OD, 300 MHz)

C	δ (^{13}C), $CDCl_3$	δ (^{1}H), $CDCl_3$
1	128.299	
2 and 6	132.195	7.812 (2H, d, J = 8.541 Hz)
3 and 5	115.556	6.949 (2H, d, J = 8.541 Hz)
4	163.226	
7	191.584	9.881 (1H, s)

Although it is the first time that this has been described in fungi, this substance is structurally related to other previously known metabolites in the said organisms that are involved in the shikimic acid pathway, such as oxime-2-(4-hydroxyphenyl)-2-oxo acetaldehyde, a metabolite previously isolated from *P. olsonii*.[44]

Finally, D-mannitol, the last non-volatile compound, was obtained from the most polar fraction, and was characterized based on the spectroscopic data of its hexa-acetate derivative. The product was crystallized in the shape of transparent hexagonal crystals. The melting point of the mix (with authentic mannitol acetate) was 123 °C–124 °C and the optical rotation was $[\alpha]_D^{20} = +23.68$ ($CHCl_3$, c 1.93), which are consistent with the literature.[45]

Polyhydroxy alcohols or sugar alcohols are biomolecules produced by many organisms, including plants, bacteria, and fungi.[46] In fungi, mannitol is the most common polyol found in large quantities in spores, fruiting bodies, sclerotia, and mycelia.[47] In *Aspergilus niger* conidiophores, for example, this compound may make up 10%–15% of the dry weight.[48] Thus, the use of raw materials derived from renewable sources remains an excellent choice for the development of new high value-added substances.

Mannitol is produced commercially by catalytic hydrogenation of fructose syrups or by inverting sugar with the co-production of another sugar alcohol sorbitol. Typically, hydrogenation of a 50/50 fructose/glucose mixture results in a 30/70 mixture of mannitol and sorbitol.[49] For better yield, some alternative processes based on the use of microbes have been suggested in the literature. For example, yeast, fungi, and lactic acid bacteria in particular are known to produce mannitol effectively without the co-formation of sorbitol.[50]

Thus, the production of mannitol by fermentation from alternative sources such as the filamentous fungi *P. roqueforti* could have interesting applications because mannitol has widespread use in clinical medicine,[51] with applications in the food and cosmetics industries.[52]

Absence of mycotoxins

Strain development of filamentous fungi has focused both on productivity and safety, and the latter is widely exploited as factories of cells in the food and beverage industry worldwide.[53] In some cases, related strains may produce toxins because of which *Penicillium* mycotoxins have been well documented.[54] However, because of the use of the present methodology for biomass production, it was not possible to detect potential bioactive toxic compounds in the mycelia of *P. roqueforti* nor in the intermediary metabolites involved in a biogenetic route that could produce these substances. Consistent with this observation, no non-volatile organic compound was found that could bind biogenetically with the mycotoxins such as patulin, PR toxin, and mycophenolic acid[54,55] that have been previously described in the literature for *Penicillium* sp. Moreover, there are no indications that the studied strain may produce botryodiplodin, a mycotoxin described by Moreau *et al*.[56] in a *P. roqueforti* strain that did not produce the PR-toxin. However, such volatile compounds were found in the NIST and Wiley mass spectral database using the Varian Saturn GC–MS equipment. This suggests the potential use of this strain in food (both animal and human).

CONCLUSIONS

In all, 50 volatile compounds were identified in the *P. roqueforti* mycelia, which included *n*-alkanes; 1-alkenes; 1-alkanols; 2-alkyl-1--alkanols; saturated and unsaturated free fatty acids; fatty acid amides; saturated and unsaturated fatty acid methyl and ethyl esters; unsaturated triglycerides and diglycerides; unsaturated monoglycerides; wax esters; lipid catabolites; mono-, sesqui-, and straight-chain terpenes; and an aromatic hydrocarbon from the shikimic acid route.

Five non-volatile compounds were identified, which have been described in *P. roqueforti* for the first time, namely, triolein, ergosterol peroxide, 9(11)-DEPH, 4-hydroxybenzaldehyde, and D-mannitol.

In summary, an unusual strain of *P. roqueforti* that did not produce any toxins was found. This study allowed identification of compounds that were already reported in the literature, but it also detected new compounds for this fungus. The cultured strain of *P. roqueforti* does not contain any component of the volatile chemical components involved in the biogenesis of PR-toxin, which is consistent with the chemical tracers proposed by Demyttenaere *et al*.,[17] Jelen,[18] and Calvert *et al*..[19] Moreover, there are no indications the studied strain may produce botryodiplodin, the mycotoxin described by Moreau *et al*.[56] in a *P. roqueforti* strain that does not produce the PR-toxin.

The results of this study support the idea that the metabolic profiles of the mycelia of this *P. roqueforti* strain can be potentially used as therapeutic agents and natural sources for the production of nutraceuticals and functional foods. Although relatively closely studied, there is no doubt that these halotolerant filamentous fungi still represent an intriguing area of research for the production of new, high-value biomolecules.

SUPPLEMENTARY MATERIAL

A detailed descriptive mycelial study together with the ^{1}H and MS spectra of the compounds **15**, **26**, **27**, **28**; ^{13}C and TOCSY spectra of the compound **26**; HSQC and HMBC spectra of the compounds **27** and **28**; and GC–MS fingerprint of the compounds **5** (n = 12, 13, and 18) and **26** can be seen at http://quimicanova.sbq.org.br, in PDF file, with free access. The GC–MS spectra of additional compounds and further spectroscopy data are available on request.

ACKNOWLEDGMENTS

The authors would like to thank the CAPES agency (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior, Brazil) for the

PhD fellowship and the European Commission for a Marie Curie Training Site Fellowship, both granted to Roberto Mioso. We gratefully acknowledge the financial support given to the project SI-697 (ULPAPD-08/01-5) by the Canary government (Agencia Canaria de Investigación, Innovación y Sociedad de la Información, ACIISI) and the ICIC (Instituto Canario de Investigación del Cáncer).The authors also express their thanks to Margaret Hart for revising the English of this manuscript.

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