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

Roberto Mioso*, Francisco J. T. Marante#, Irma H. Bravo de Laguna#, Juan E. G. González* and Juan J. S. Rodríguez*
Departamento de Química, Universidad de Las Palmas de Gran Canaria, Gran Canaria 35017, Spain
Departamento de Biología, Universidad de Las Palmas de Gran Canaria, Gran Canaria 35017, Spain
Departamento de Ingeniería de Procesos, Universidad de Las Palmas de Gran Canaria, Gran Canaria 35017, Spain

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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 α-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. 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.

Although it is described as a terrestrial fungus, some studies have shown that P. roqueforti strains have high salt tolerance, with spore germination inhibited only at sodium chloride concentrations of over 100 g L⁻¹. This extraordinary tolerance to salt, along with its high osmotic pressure, indicates that this hyphomycete adapts to life as a marine facultative fungus. 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, some of which have antiparasitic and bacteriostatic properties. Its proteolytic enzymes allow it to be applied in various processes, such as removal of scales of some fish, in the fishing industry and in biotechnology for the production of high-quality fat from food waste.

With regard to the P. roqueforti chemical composition, it is worth noting the extensive research performed on its fatty acid profile and lipid metabolism. 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, and the free steroids have an ergosterol skeleton. Further data are available on the biogenesis of unsaturated fatty acids depending on the phases of growth.

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

P. roqueforti produces several mycotoxins, such as PR-toxin, roquefortine C, mycophenolic acid, patulin, and penicillic acid, some of which are known to be generally unstable or incapable of causing serious damage at low concentrations. 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. PR-toxin is the most significant because it is reported to cause damage to the liver and kidney in rats and is also potentially carcinogenic. However, it should be noted that fungal metabolite production depends on several variables such as the isolate, growth medium, and environmental factors.

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. 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.

*Email: robermotioso@yahoo.co.uk
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 10 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 (Buchi 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 CH₃Cl/CH₃OH (50:50, 2 h). The extracts were applied at the top of the column and eluted with CH₃Cl/CH₃OH (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 Analytica) 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-18F₂₅₄ plates (0.25 mm; Merck) using CH₃CN/CH₃OH/H₂O (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 low-pressure chromatography system also based on the Fluid Metering Inc. apparatus.

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 CH₂Cl₂ (×3, 24 h) and CH₃OH (×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. 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 (11-14) fatty acid methyl and ethyl esters; unsaturated triglycerides (15) and diglycerides (16); unsaturated...
<table>
<thead>
<tr>
<th>N°</th>
<th>R&lt;sub&gt;t&lt;/sub&gt; (min) (mean ± SD)</th>
<th>Compound (structure&lt;sup&gt;1&lt;/sup&gt;); concentration (mg x kg&lt;sup&gt;−1&lt;/sup&gt;)</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>12.075 ± n.d.</td>
<td>Dodecane (1, n = 9); 0.004</td>
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<tr>
<td>2</td>
<td>12.437 ± n.d.</td>
<td>1-Dodecanol (3, n = 10); 0.002</td>
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<td>3</td>
<td>12.524 ± 0.007</td>
<td>2-Butyl-1-octanol (4, n = 3, m = 3); 4.3</td>
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<td>4</td>
<td>13.235 ± n.d.</td>
<td>Tetradecane (1, n = 11); 0.07</td>
</tr>
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<td>13.252 ± n.d.</td>
<td>2-Hexyl-1-octanol (4, n = 3, m = 5); 0.6</td>
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<tr>
<td>6</td>
<td>13.454 ± n.d.</td>
<td>Benzaldehyde, 4-hydroxy (26); 1.1</td>
</tr>
<tr>
<td>7</td>
<td>13.684 ± 0.004</td>
<td>Non-anionic acid, 9-oxo-, methyl ester (21); 39.7</td>
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<td>8</td>
<td>13.972 ± n.d.</td>
<td>1-Pentadecene (2, n = 12); 1.1</td>
</tr>
<tr>
<td>9</td>
<td>14.005 ± n.d.</td>
<td>Geranyl isovalerate (23); 114.4</td>
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<td>10</td>
<td>14.471 ± n.d.</td>
<td>1-Dodecanol, 3,7,11-trimethyl (24); 3.78</td>
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<td>12</td>
<td>14.771 ± 0.005</td>
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<td>14.843 ± 0.002</td>
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<td>14</td>
<td>14.857 ± 0.010</td>
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<td>15</td>
<td>15.642 ± 0.000</td>
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<td>15.906 ± n.d.</td>
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<td>16.338 ± 0.010</td>
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<td>20</td>
<td>16.348 ± 0.022</td>
<td>1-Eicosanol (3, n = 18); 20.0</td>
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<td>16.501 ± n.d.</td>
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<td>23</td>
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<td>24</td>
<td>16.604 ± 0.020</td>
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<td>25</td>
<td>16.711 ± n.d.</td>
<td>2-Pentadecanone, 6,10,14-trimethyl- (25); 15.9</td>
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<td>26</td>
<td>17.160 ± 0.015</td>
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<td>27</td>
<td>17.273 ± 0.006</td>
<td>Hexadecanoic acid, methyl ester (6, n = 14); 1884.1</td>
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<tr>
<td>28</td>
<td>17.289 ± 0.019</td>
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<tr>
<td>29</td>
<td>17.928 ± 0.020</td>
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<td>30</td>
<td>18.352 ± n.d.</td>
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<td>31</td>
<td>18.385 ± 0.004</td>
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<td>32</td>
<td>18.389 ± n.d.</td>
<td>Triolein (15); 2.9</td>
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<td>33</td>
<td>18.394 ± 0.009</td>
<td>8,11-Octadecadienoic acid, methyl ester (11, n = 5, m = 6); 100.8</td>
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<td>34</td>
<td>18.550 ± 0.028</td>
<td>Octadecanoic acid, methyl ester (6, n = 16); 60.7</td>
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<td>18.558 ± n.d.</td>
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<td>36</td>
<td>18.811 ± n.d.</td>
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<td>37</td>
<td>18.962 ± n.d.</td>
<td>9-Octadecenoic acid (Z)-, tetradecyl ester (20; n = 7); 65.0</td>
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<tr>
<td>38</td>
<td>19.817 ± 0.001</td>
<td>9-Octadecenoic acid (oleic) acid (Z)-, tetradecyl ester (20; n = 7); 65.0</td>
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<td>39</td>
<td>19.818 ± 0.004</td>
<td>13-Docosenoic (erucic) acid, (Z)- (8, n = 7, m = 11); 10.3</td>
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<td>40</td>
<td>19.857 ± 0.001</td>
<td>Hexadecanoic acid, methyl ester (6, n = 14); 1884.1</td>
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<td>41</td>
<td>19.897 ± 0.005</td>
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<td>43</td>
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<td>22.143 ± n.d.</td>
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<td>22.486 ± n.d.</td>
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<td>49</td>
<td>25.823 ± n.d.</td>
<td>Docosanoic acid, methyl ester (6, n = 20); 12.7</td>
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</table>

<sup>1</sup>“Structure” refers to the compound number in figures; R<sub>t</sub> = Retention time; n.d. = no date.
Biomolecules produced in liquid-state fermentation by a marine-derived fungus, *Penicillium roqueforti*

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).

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

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. 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.

**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*

Non-volatile organic compounds

Non-volatile compounds obtained from *P. roqueforti* mycelia were fractionated and measured by gravimetric analysis and integration
of the 1H-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)-dehydroergosterol peroxide (DHEP; 28, 2.15%), 4-hydroxybenzaldehyde (26, 0.92%), unidentified phospho- and glycolipids (0.31%), mannnitol (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 mannnitol, 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, 1H-NMR, 13C-NMR, 1H-1H-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, 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, 1H-NMR, 13C-NMR, 1H-1H-(COSY, TOCSY, NOESY), DEPT_135, HSQC, HMBC, and mass spectroscopic data.

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 positions or the percentage of total lipids found in fungi, working with a sterol mixture from the marine sponge Tethya aurantia suggested that EP was formed by biological processes.

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

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 171–176 °C [Mp = 171 °C (CHCl3, c 1.2), and was identified using IR, 1H-NMR, 13C-NMR, 1H-1H-(COSY, TOCSY, NOESY), DEPT_135, HSQC, HMBC, and mass spectroscopic data. The 1H-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.

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. and Mediavilla (Table 1S in the supplementary material) showed a linear relationship between the value of [α]D and the percentage of EP, where

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

**Table 2. Ergosterol peroxide (27) spectra (13C-NMR and 1H-NMR, CDCl3, 400 MHz)**

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<th>C</th>
<th>δ (13C)</th>
<th>δ (1H)</th>
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<tbody>
<tr>
<td>1</td>
<td>34.683</td>
<td>1.0-2.2 (20 H, m)</td>
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<td>2</td>
<td>30.106</td>
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<tr>
<td>3</td>
<td>66.465</td>
<td>3.93 (m, 1H)</td>
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<td>4</td>
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<td>82.135</td>
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<tr>
<td>21</td>
<td>42.760</td>
<td></td>
</tr>
<tr>
<td>22</td>
<td>33.053</td>
<td></td>
</tr>
<tr>
<td>23</td>
<td>19.932</td>
<td>0.772 (3H, d, J = 6.8 Hz)</td>
</tr>
<tr>
<td>24</td>
<td>20.617</td>
<td>0.788 (3H, d, J = 6.4 Hz)</td>
</tr>
<tr>
<td>25</td>
<td>17.547</td>
<td>0.863 (3H, d, J = 6.8 Hz)</td>
</tr>
<tr>
<td>26</td>
<td>51.671</td>
<td></td>
</tr>
<tr>
<td>27</td>
<td>29.686</td>
<td></td>
</tr>
<tr>
<td>28</td>
<td>39.335</td>
<td></td>
</tr>
</tbody>
</table>

**Figure 4. Non-volatile compounds identified in Penicillium roqueforti**
Biomolecules produced in liquid-state fermentation by a marine-derived fungus, *Penicillium roqueforti*

By introducing the value \([\alpha]_{20}^0 = -9.7\) 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 \(\delta\) 130.970 (δ 51.081 in EP) and C-11 carbon at \(\delta\) 125.00 (δ 20.866 in EP). Moreover, the \(^1\)H-NMR spectrum of this mixture gives a signal at \(\delta\) 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,OD, 300 MHz)

<table>
<thead>
<tr>
<th></th>
<th>(\delta) (ppm) (^{13})C</th>
<th>(\delta) (ppm) (^1)H</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>128.299</td>
<td></td>
</tr>
<tr>
<td>2 and 6</td>
<td>132.195</td>
<td>7.812 (2H, d, J = 8.541 Hz)</td>
</tr>
<tr>
<td>3 and 5</td>
<td>115.558</td>
<td>6.949 (2H, d, J = 8.541 Hz)</td>
</tr>
<tr>
<td>4</td>
<td>163.226</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>191.584</td>
<td>9.881 (1H, s)</td>
</tr>
</tbody>
</table>

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, \(\alpha\)-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 \([\alpha]_{20}^0 = +23.68\ (CHCl_3, c 1.93)\), 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 *Aspergillus 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\(^{44,55}\) 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 \(\alpha\)-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 \(\alpha\)-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.\(^{57}\), Jelen,\(^{58}\) 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
REFERENCES


Biomolecules produced in liquid-state fermentation by a marine-derived fungus, *Penicillium roqueforti*


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

Roberto Mioso*, Francisco J. T. Marantea, Irma H. Bravo de Lagunaa, Juan E. G. Gonzáleza and Juan J. S. Rodríguezc

dDepartment of Chemistry, University of Las Palmas de Gran Canaria, Gran Canaria 35017, Spain
dDepartment of Biology, University of Las Palmas de Gran Canaria, Gran Canaria 35017, Spain
cDepartment of Process Engineering, University of Las Palmas de Gran Canaria, Gran Canaria 35017, Spain

Table 1S. Optical rotation described to Ergosterol Peroxide (27) and 9(11)-dehydroergosterol peroxide (28)

<table>
<thead>
<tr>
<th>Compounds mixture (EP: DHPE, %)</th>
<th>[α]D20°</th>
</tr>
</thead>
<tbody>
<tr>
<td>0:100 (DHEP pure)</td>
<td>+80.0</td>
</tr>
<tr>
<td>Experimental P. roqueforti mixture</td>
<td>-9.7</td>
</tr>
<tr>
<td>81.5:18.5 (Mediavilla)68</td>
<td>-12.5</td>
</tr>
<tr>
<td>84:16 (Fisch et al.)77</td>
<td>-14.2</td>
</tr>
<tr>
<td>100:0 (EP pure)</td>
<td>-32.9</td>
</tr>
</tbody>
</table>

Figure 1S. Identification report of the fungal strain by CABI Bioscience, Surrey, UK
Descriptive mycelial study

89.9 g of dried mycelium was obtained by maceration extraction in CH₂Cl₂ (x3, 24 h each one, at room temperature). After the filtration, evaporation and drying (vacuum), this gave 14.804 g of raw extract. The 1H-NMR spectrum of this mixture of substances revealed the presence of aromatic protons at δ 8.4-7.2, olefinic protons at δ 6.6-5.0, geminal to heteroatom protons at δ 4.0-3.2, and typical hydrocarbon chain protons at δ 3.0-0.8.

This raw extract was subjected to the partitioning scheme described in Figure 2S. Thus, it was dissolved in 200 ml of CH₂Cl₂ and was partitioned in a separatory decantation funnel with an additional 200 mL of H₂O. The organic phase was separated and the aqueous phase re-extracted with CH₂Cl₂. The organic phases were combined to yield 1.637 g of the crude liposoluble fraction (Prm-L-0).

The 1H-NMR spectrum of this mixture revealed the presence of olefinic protons at δ 5.8-5.0, geminal to heteroatom protons at δ 4.0-3.0 and typical hydrocarbon chain protons at δ 3.0-0.8. Finally, from the liposoluble fraction, three different sub-fractions were obtained: Prm-L-1, Prm-L-2 and Prm-L-3. The aqueous phase was re-extracted with 2-butanol to give the "hidrosoluble (Prm-H-0) fraction”.

Study of the liposoluble-1 fraction (Prm-L-1)

540 mg of a colourless oil was produced that, on TLC analysis, was seen to be a mixture of five major substances. The following volatile substances were identified by GC-MS:

- Hexadecanoic acid, methyl ester (6; n= 14; Rt= 17.302; 12.16 mg)
- Heptadecanoic acid, methyl ester (6; n= 15; Rt= 17.942; 0.44 mg)
- 8,11-Octadecadienoic acid, methyl ester (11; n= 5, m= 6; Rt= 18.403; 8.060 mg)

The product weighed a total 409.24 mg that corresponded to non-volatile material formed by components that did not volatilize at the injector temperature used, or were outside the predetermined scanning time in the method. The 1H-NMR spectrum detected an AB system at δ 6.8-6.0, olefinic protons (δ 5.6-5.0), geminal to heteroatom protons (δ 4.1-3.0) and typical protons of hydrocarbon chains (δ 3.0-0.5). The 13C-NMR spectrum showed carbonyl carbons (δ 178-173), olefinic carbons (δ 142-116), geminal to heteroatom carbons (δ 83-60) and saturated carbons typical of hydrocarbon chains (δ 56-11). This suggests the presence of ergosterol peroxide and unsaturated triglycerides.

It was filtered through lipophilic Sephadex LH-20 eluting with CH₂Cl₂: MeOH (1: 1), which gave three homogeneous fractions by TLC: Prm-L-1-a (290 mg); Prm-L-1-b (105 mg); and Prm-L-1-c (110 mg).

The Prm-L-1-a mixture was fractionated by semi-preparative HPLC (normal phase, Hex: EtOAc, 80: 20) to give three sub-fractions: Prm-L-1-a-1, Prm-L-1-a-2 and Prm-L-1-a-3. All of these were analyzed by NMR and GC-MS as described below.

Prm-L-1-a-1
This gave 45 mg of an oil in which the following volatile substances were identified by GC-MS:

- Octadecane (1; n= 15; Rt= 16.418; 0.2 mg)
- Hexadecanoic acid, methyl ester (6; n= 14; Rt= 17.297; 1.891 mg)
- Heptadecanoic acid, methyl ester (6; n= 15; Rt= 17.933; 0.12 mg)
- 8,11-Octadecadienoic acid, methyl ester (11; n= 5, m= 6; Rt= 18.391; 3.62 mg)
- Heptadecanoic acid, 16-methyl-, methyl ester (9; n= 13; Rt= 18.558; 1.04 mg)
- 9-Octadecenoic (oleic) acid (Z)-, tetradecyl ester (20; n= 7; Rt= 19.817; 0.47 mg)

This gave a total of 36.039 mg of non-volatile material, presumably formed by components that did not volatilize at the injector temperature used, or that were outside the pre-determined scanning time in the method. This material was identified, by integrating the 1H-NMR spectrum, as a mixture (1.0: 1.6) of ergosterol peroxide (27) (δ 6.55-6.14 (AB system characteristic)) and unsaturated triglyceride triolein type (15) (δ 5.28 (m); δ 4.34-4.01 (two characteristic dd); δ 2.79 (t); δ 2.31 (t); δ 2.04 (m); δ 1.63 (m); δ 1.29 (m); δ 0.92 (t)).

Prm-L-1-a-2
This gave 187 mg of an oil in which the following volatile substances were identified by GC-MS:

- Nonanoic acid, 9-oxo-, methyl ester (21; Rt= 13.687; 3.42 mg)
- Tetradecanoic acid, methyl ester (6; n= 12; Rt= 15.898; 0.07 mg)
- Pentadecanoic acid, methyl ester (6; n= 13; Rt= 16.598; 1.57 mg)
- Hexadecanoic acid, methyl ester (6; n= 14; Rt= 17.270; 154.73 mg)
- 11-Octadecenoic acid, methyl ester (8; n= 5, m= 9; Rt= 18.389; 25.38 mg)

This weighed a total 1.60 mg of non-volatile components that did not volatilize at the injector temperature used or that were outside the pre-determined scanning time in the method. Using 1H-NMR, this was identified as ergosterol peroxide (27) (δ 6.55-6.14) characteristic dd of the AB system produced by the vinyl protons at C-6 and C-7).
This gave 53 mg of a yellow oil in which the following volatile substances were identified by GC-MS:
1-Dodecanol (3; n= 10; Rt= 12.437; 0.0002 mg)
1-Tridecanol (3; n= 11; Rt= 14.847; 0.2912 mg)
1-Hexadecanol (3; n= 14; Rt= 16.329; 0.3238 mg)
Triolein (15; Rt= 18.389; 0.26 mg)
Eicosanoic acid (5; n= 18; Rt= 18.962; 2.78 mg)
1-Docosanol (3; n= 20; Rt= 22.482; 2.28 mg)

It gave 47.065 mg of non-volatile components that did not volatilize at the injector temperature used or that were outside the pre-determined scanning time in the method. Using 1H-NMR, this was identified as a mixture of unsaturated triglycerides similar to triolein (15) (δ 5.52 (m); δ 4.35-3.90 (two characteristic dd); δ 2.78 (t); δ 2.33 (t); δ 2.04 (m); δ 1.62 (m); δ 1.29 (m); δ 0.91 (m)). The 13C-NMR spectrum also supports the carbonyl carbon allocation at δ 173.979-173.948; olefinics at δ 130.054-127.927; geminal to oxygens at the structural sub-unit of the glycerol (δ 72.124-62.001), and the same for the hidrocarbon aliphatic chains at δ 34.286-14.163.

Via re-chromatography over silica gel with hexane-ethyl acetate (98: 2), some 2.17 mg of a homogeneous oil was obtained by TLC, the spectroscopic data of which were consistent with the structure of triolein (15):
1R (CHCl3) - 3029.48; 3006.99; 1738.68; 1653.08; 1232.81; 1168.45 cm⁻¹
1H-NMR (CDCl3) - δ 5.34 (6H, t, J= 5.6 Hz); 5.27 (1H, dd, J= 4.3 Hz; J= 5.9 Hz); 4.30 (2H, dd, J= 4.3 Hz; J= 11.9 Hz); 4.14 (2H, dd (J= 5.9 Hz; J= 11.9 Hz); 2.30 (6H, t, J= 7.5 Hz); 2.01 (12H, m); 1.61 (6H, m); 1.27 (60H, m); 0.89 (9H, t, J= 6.6 Hz)).
MS, m/z (%) - 603.5567 (M⁺-197 (98.16 %); 602.5514 (100 %); 265.2749 (13.4 %); 55.0413 (100 %).
13C-NMR (300 MHz, CDCl3) - δ 14.018; 22.606; 24.767; 27.087; 27.130; 29.022; 29.094; 29.248; 29.454; 29.623; 29.684; 31.835; 33.930; 34.092; 62.006; 68.810; 129.592; 129.892; 172.723; 173.132; 173.73.

The Prm-L-1-b-1 fraction (105 mg) was a mixture of at least six substances, a fact that was revealed by analytical TLC. However, using HPLC, ten major fractions were separated which of only the following volatile components were identified by GC-MS:
Hexadecanoic acid, methyl ester (6; n= 14; Rt= 17.288; 5.01 mg)
Octadecanoic acid, methyl ester (6; n= 16; Rt= 18.554; 0.67 mg)

The fractionation was carried out by semi-preparative HPLC (normal phase, Hex: EtOAc, 80: 20) to give the following sub-fractions: Prm-L-1-b-1-1 and Prm-L-1-b-1-2.

This gave 78 mg of a semi-solid material which GC-MS analysis allowed to identify as the following volatile substances:
1-Hexadecane (2; n= 13; Rt= 14.845; 0.96 mg)
Pentadecanoic acid, methyl ester (6; n= 13; Rt= 16.584; 0.67 mg)
2-Pentadecanone, 6,10,14-trimethyl- (25; Rt= 16.711; 1.43 mg)
9-Hexadecanoic acid, methyl ester, (Z)- (8; n= 5, m= 7; Rt= 17.145;
34.08 mg)
7,10,13-Eicosatrienoic acid, methyl ester (13; Rt= 19.542; 12.6 mg)
6,9,12,15-Docosatetraenoic acid, methyl ester (14; Rt= 20.205; 7.73 mg)
9,12-Eicosadienoic (linoleic) acid (Z,Z)-, ethyl ester (12; Rt= 21.454; 6.18 mg)
9,12-Octadecadienoic (linoleic) acid (Z,Z)-, 2,3-dihydroxypropyl ester (18; Rt= 22.143; 4.4 mg)

This consisted of 110 mg of an oily material which was shown by analytical TLC to be a mixture of six substances. A GC-MS analysis

The total weight was 8.35 mg of non-volatile material that, as before, did not volatilize at the injector temperature used, or were outside the pre-determined scanning time for the method. A study of the integral curve of the 1H-NMR spectrum of this fraction indicated that it was a mixture 2.3: 0.4 of ergosterol peroxide (27), and 9(11)-dehydroergosterol peroxide (28) (δ 6.55-6.20 (two characteristic AB systems slightly offset from one another); δ 5.43-5.10 (m, vinyl protons); δ 3.96 (m, characteristic of H geminal to hydroxyl at C-3); δ 2.14-0.60 (m, CH₃; and CH₂). These deductions are confirmed by the 13C-NMR spectrum, wherein the following is observed: carbonyl carbons (δ 179.440), aromatics (δ 167.800), olefins (δ 135.000-126.000); geminal to oxygen atoms (δ 82.000-65.000); and methine/ methylene /methyls (δ 55.000-12.500).
identified/quantified these volatile components: 2-Butyl-1-octanol (4; n= 3, m= 3; Rt= 12.528; 0.14 mg) 2-Hexadecan-1-ol (4; n= 3, m= 5; Rt= 13.252; 0.055 mg) 1-Dodecan-3,11-trimethyl (24; Rt= 14.471; 0.34 mg) 2-Hexyl-1-decanol (4; n= 5, m= 5; Rt= 15.647; 0.131 mg) Hexadecanoic acid, methyl ester (6; n= 14; Rt= 17.290; 5.78 mg) Octadecanoic acid, methyl ester (6; n= 16; Rt= 18.552; 0.86 mg) 13-Docosenoic (erucic) acid, (Z)- (8; n= 7, m= 11; Rt= 19.815; 0.3 mg)

This gave 93.936 mg of non-volatile material that was fractionated by semi-preparative HPLC into two fractions: fraction Prm-L-1-c-1 and fraction Prm-L-1-c-2.

**Prm-L-1-c-1**

This consisted of 55 mg of a white solid substance which was identified again as ergosterol peroxide (27) on account of its physico-chemical constants and spectroscopic data: M.P. = 178-180 ºC (crystallized from methanol) [α]25⁰C (CHCl₃, c 1.24) = -25º and fraction Rp= 20ºC (CHCl₃) - See table 2.

**Prm-L-1-c-2**

This gave 50 mg of a semi-solid substance with some minor volatile components identified by GC-MS: 1-Pentadecane (2; n= 12; Rt= 13.972; 0.098 mg) 1-Eicosanol (3; n= 18; Rt= 16.327; 0.073 mg) 9-Octadecenoic acid (Z)-, 9-octadeceyl ester, (Z)- (19; Rt= 19.769; 0.533 mg)

This weighed a total 48.666 mg of non-volatile material that did not volatilize at the injector temperature used or that were outside the pre-determined scanning time in the method. A study of the 1H-NMR spectrum (δ 1.92-0.73) indicated that it was a mixture of high molecular weight alkanes.

**Prm-L-2-a**

This gave 35 mg of an oily material in which only one volatile component present at the trace level was identified by GC-MS: 13-Docosenoic (erucic) acid, (Z)- (8; n= 7, m= 11; Rt= 19.816; 0.034 mg)

It gave 34.918 mg of non-volatile material that did not volatilize at the injector temperature used or that was outside the pre-determined scanning time in the method. A study of the 1H-NMR spectrum (δ 1.92-0.73) indicated that it was a mixture of high molecular weight alkanes.
It gave 29.94 mg of non-volatile material that did not volatilize at the injector temperature used, or that were outside the pre-determined scanning time in the method. By 1H-NMR spectroscopy, this was revealed to be a mixture of steroidal unsaturated waxes (δ 5.56-5.03 (C=CH)); 4.81-4.57 (COOCH); 2.84-2.64 (C=C-CH=C=C); 2.15-1.40 (CH₂COO, CH and CH₃ of steroid skeletons); 1.38-1.11 (CH₃, intense, long hydrocarbon chains); 1.04-0.72 (CH₃, intense, angular methyl sterol skeletons)]. The attempts to separate these substances were unsuccessful.

Prm-L-2-c
This gave 109 mg of a semi-solid material with the following volatile substances as identified by GC-MS analysis:
1-Eicosanol (3; n= 18; Rt= 16.358; 0.0093 mg)

This means that this fraction contains 107.1924 mg of non-volatile components that did not volatilize at the temperature used for the injector or that exceeded the pre-determined scanning time in the method. Two subfractions were obtained by normal phase semi-preparative HPLC: Prm-L-2-c-1 and Prm-L-2-c-2.

Prm-L-2-c-1
This gave 10 mg of an oily material, the 1H-NMR spectrum of which revealed signals indicative of waxes structurally similar to those identified by GC-MS in other fractions (δ 5.57-5.26 (C=CH); 4.81-4.54 (COOCH); 2.83-2.69 (C=C-CH=C=C); 2.21-1.48 (CH₂CO, C=C-CH₃); 1.43-1.19 (CH₃, intense, long hydrocarbon chains); 1.01-0.74 (terminal CH₃)].

Prm-L-2-c-2
This gave 83 mg of a semi-solid material with only one volatile substance identified by GC-MS analysis:
1-Eicosanol (3; n= 18; Rt= 16.357; 1.66 mg)

This gave 82.961 mg of non-volatile material that did not volatilize at the injector temperature used or that were outside the pre-determined scanning time in the method. This component was identified by the 1H-NMR spectrum as ergosterol peroxide (27) (δ 6.57-6.17 (2H, characteristic AB system, CH=CH); 5.21 (2H, m, C=CH); 4.10 (1H, m, CH-CH₂); 2.17-1.10 (m, CH and CH₃ of the steroid skeleton); 1.09-0.72 (angulars CH₃)].

Prm-L-2-d
This gave 105 mg of a viscous oil which revealed the following volatile components by GC-MS analysis:
Benzaldehyde, 4-hydroxy (26; Rt= 13.454; 0.1 mg)
1-Eicosanol (3; n= 18; Rt= 16.352; 0.062 mg)
Pentadecanoic acid, methyl ester (6; n= 13; Rt= 16.622; 0.036 mg)
Hexadecanoic acid, methyl ester (6; n= 14; Rt= 17.290; 1.97 mg)
9-Octadecanamide (10; Rt= 18.366; 0.85 mg)
8,11-Octadecadienoic acid, methyl ester (11; n= 5, m= 6; Rt= 18.389; 1.0 mg)
Octadecanoic acid, methyl ester (6; n= 16; Rt= 18.556; 0.54 mg)
Trijcosane (1; n= 20; Rt= 19.753; 0.53 mg)
9-Octadecenoic (oleic) acid (Z)-, tetradecyl ester (20; n= 7; Rt= 19.817; 0.86 mg)

This revealed a total of 98.99 mg of non-volatile material, the components of which did not volatilize at the injector temperature used or that were outside the pre-determined scanning time in the method. By 1H-NMR spectrum analysis, a mixture made up of phenol aldehydes was determined (δ 12.29; 12.10; 9.88; 7.91-7.50; 7.15-6.67) together with waxes. This was purified by low pressure CC (silica gel, hexane-ethyl acetate, 90:10) that yielded the fraction Prm-L-2-d-1 (25 mg), proved to be homogeneous by TLC. This was crystallized from methanol to give colorless prisms of 4-hydroxy-benzaldehyde (26):
M.P. - 118-119 ºC (Methanol).
I.R (CHCl₃) - 3590.81; 3338.71; 3002.84; 1678.99; 1604.06; 1586.85; 1511.54; 1442.68; 1274.83; 1223.19; 1156.48; 1102.69; 859.3; 838.01 cm⁻¹.
1H-NMR - See table 3.
13C-NMR - See table 3.
MS, m/z (%): 106.0794 (M⁺; 2.5 %); 105.0747 (M⁺-H; 16.7 %); 79.0599 (52.61 %);
78.0516 (M⁺-CO; 25.43 %); 77.0444 (M⁺-H-CO; 72.4 %).

Study of the liposoluble fraction-2 (Prm-L-3)
This gave 380 mg of a viscous oil which 1H-NMR spectrum (DOCD) showed to be one or more polyhydroxy compounds [δ 5.16 (1H, s wide; 4.05-3.35 (6H, m)]. This material was purified by reverse-phase semi-preparative HPLC, resulting in the fraction Prm-L-3-1 (310 mg). This was also analyzed by GC-MS, allowing us to identify the following volatile substance:
1-Eicosanol (3; n= 18; Rt= 16.357; 1.66 mg)

There were 305.45 mg of non-volatile material, the components of which did not volatilize at the injector temperature used or were outside the pre-determined scanning time in the method. By 1H-NMR spectrum analysis this seemed to be made up of one or more monosaccharides in the form of α-pyranose [δ 5.10 (1H, d, J= 3.6 Hz, CH anomeric); 3.83-3.21 (9H, m, CH=C=CH₂ geminals to OH groups)]; the 13C-NMR spectrum (DOCD) also confirmed that deduction (δ 94.948 for the anemic carbon and 74.442-54.716 for the other carbons).
Through an analysis of this fraction by HPLC with a Shodex OH Pak SB806 HQ column thermostated at 30 °C (water 0.05% NaN₃ as eluent at a flow of 1.0 ml/ min and a refractive index detector), and after filtration through a Sep-Pak C₁₈ of Water cartridge, this was found to be made up of two components, a minor component (30.63%) with a retention time (Rt= 11.481 min) matching that of the D-(+)-mannitol [a mixed injection of the Prm-L-3-1 fraction with an authentic sample of D-(+)-mannitol had the same chromatogram with the peak of the minor substance (Rt= 11.481) increased in intensity (57.38%)], and another major substance (69.11%) with a retention time (Rt= 12.711) which did not coincide with authentic samples of commercial monosaccharides as D-(+)-Glucose (Rt= 11.481) or D-(+)-mannose (Rt= 11.188). A more detailed study of this gluco-pyranose will be published shortly.

Study of the hidrosoluble fraction (Prm-H-0)
The fraction which was obtained directly from the resultant aqueous phase by separating the liposoluble element from the crude extract, was re-extracted (equal volume, x1) with 2-butanol. The evaporation of the solvent gave 1761 mg of a viscous oil (Prm-H-0) which was shown to be “highly polar” by thin layer chromatography in its normal phase. In the 1H-NMR spectrum of this crude fraction, geminal to heteroatom protons were observed (δ 3.83-3.21) together with the typical unsaturated hydrocarbon chains (δ 5.32 and 2.31-0.79). The 13C-NMR spectrum shows olefinic carbons at δ 130.240-127.865; geminal to heteroatoms at δ 77.590-54.517 and typical aliphatic hydrocarbon chains (δ 31.507-9.598). It follows, therefore, that there is probably presence of a mixture of sugars with impurities from the previous lipid fractions. Indeed, by GC-MS, the following volatile organic components were detected:
Geranyl isovalerate (23; Rt= 14.005; 10.28 mg)
9-Octadecenoic (oleic) acid (Z)-, tetradecyl ester (20; n= 7; Rt= 19.818; 4.98 mg)
Pentacosane (1; n= 22; Rt= 21.820; 2.86 mg)

Two subfractions were separated by semi-preparative reverse phase HPLC: Prm-H-0-1 and Prm-H-0-2.

Prm-H-0-1
This gave 8.5 mg of a viscous oil. By GC-MS, the following volatile compounds were detected:
Dodecane (1; n= 9; Rt= 12.075; 0.00032 mg)
2-Butyl-1-octanol (4; n= 3, m= 3; Rt= 12.517; 0.00053 mg)
Tetradecanoic acid (5; n= 12; Rt= 14.766; 0.00131 mg)
9-Octadecanamide (10; Rt= 18.352; 0.00475 mg)
9-Octadecenoic acid (Z)-, 9-octadecenyl ester, (Z)- (19; Rt= 19.796; 0.00401 mg)

This gave 8.49 mg of non-volatile material, the components of which did not volatilize at the injector temperature used or that were outside the pre-determined scanning time for the method. By analysis of the 'H-NMR spectrum, this appeared to be made of phospho- and glycolipids that remained unidentified.

Prm-H-0-2
This gave 1670 mg of a semi-solid material with the following volatile components detected by GC-MS:
2-Butyl-1-octanol (4; n= 3, m= 3; Rt= 12.528; 0.22 mg)
2-Hexyl-1-decanol (4; n= 5, m= 5; Rt= 15.649; 0.16 mg)
Pentadecanoic acid (5; n= 13; Rt= 15.906; 0.22 mg)
Pentadecanoic acid, 14-methyl-, methyl ester (9; n= 11; Rt= 17.279; 0.61 mg)
13-Docosenoic (erucic) acid, (Z)- (8; n= 7, m= 11; Rt= 19.810; 0.59 mg)

This gave a total of 1664.93 mg of non-volatile material that did not volatilize at the injector temperature used or that were outside the pre-determined scanning time for the method.

Figure 3S. GC-MS fingerprint of the tetradecanoic acid ethyl ester (5, n= 12)
Figure 4S. GC-MS fingerprint of the pentadecanoic acid (\(5, n=13\))

Figure 5S. GC-MS fingerprint of the eicosanoic acid methyl ester (\(5, n=18\))
Figure 6S. GC-MS fingerprint of the 4-hydroxibelzaldehyde (26)

Figure 7S. $^1$H-NMR spectrum (CDCl$_3$, 300 MHz) of the Triolein (15)
Biomolecules produced in liquid-state fermentation by a marine-derived fungus, *Penicillium roqueforti*

**Figure 8S.** MS spectrum of the Triolein (15)

**Figure 9S.** $^1$H-NMR spectrum (CDCl$_3$, 250 MHz) of the Ergosterol Peroxide (27)
Figure 10S. $^1$H-NMR spectrum (CDCl$_3$, 250 MHz) of the ergosterol peroxide (27) and its derivative, 9(11)-dehydroergosterol peroxide (28).

Figure 11S. HSQC spectrum (CDCl$_3$, 400 MHz) of the ergosterol peroxide (27) and its derivative, 9(11)-dehydroergosterol peroxide (28).
Biomolecules produced in liquid-state fermentation by a marine-derived fungus, *Penicillium roqueforti*

Figure 12S. $^1$H-NMR spectrum ($CDCl_3$, 400 MHz) of the ergosterol peroxide (27) and its derivative, 9(11)-dehydroergosterol peroxide (28)

Figure 13S. HMBC spectrum ($CDCl_3$, 400 MHz) of the ergosterol peroxide (27) and its derivative, 9(11)-dehydroergosterol peroxide (28)
Figure 14S. $^1$H-NMR spectrum (CDCl$_3$, 250 MHz) of the ergosterol peroxide (27) and its derivative, 9(11)-dehydroergosterol peroxide (28)

Figure 15S. Mass spectrum (MS) of the ergosterol peroxide (27) and its derivative, 9(11)-dehydroergosterol peroxide (28)
Biomolecules produced in liquid-state fermentation by a marine-derived fungus, *Penicillium roqueforti*

**Figure 16S.** $^{13}$C-NMR spectrum (CD$_3$OD, 300 MHz) of the 4-hydroxybenzaldehyde (26)

**Figure 17S.** TOCSY spectrum (CD$_3$OD, 300 MHz) of the 4-hydroxybenzaldehyde (26)
Figure 18S. Mass spectrum (MS) of the 4-hydroxybenzaldehyde (26)

Figure 19S. $^1$H-NMR spectrum (CD$_3$OD, 300 MHz) of the 4-hydroxybenzaldehyde (26)
Figure 20S. "\textsuperscript{1}H-NMR spectrum (CD\textsubscript{3}OD, 300 MHz) of the D-mannitol (29)"