Cryptoporic and isocryptoporic acids from the fungal cultures of *Polyporus arcularius* and *P. ciliatus*

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**Abstract**

In a chemical study of several fungal cultures of *Polyporus*, a methyl ester of cryptoporic H was isolated from *P. ciliatus*, together with cryptoporic acid H and 5-hydroxymethylfuran-3-carboxylic acid. Furthermore, two additional compounds, named isocryptoporic acids H and I, were isolated from *P. arcularius*. These isocryptoporic acids are isomers of the cryptoporic acids with drimenol instead of albicanol as the terpenoid fragment; their structural elucidation was determined by application of spectroscopic methods. © 2002 Elsevier Science Ltd. All rights reserved.

**Keywords:** *Polyporus*; Polyporales; Basidiomycete, Drimane sesquiterpenoids

1. **Introduction**

*Polyporus* species are basidiomycetes belonging to the Polyporales, which are wood-rotting fungi. They grow on living trees or dead wood, and possess exoenzymes that can degrade cellulose and lignin. Many of them, fruiting bodies collected from host trees or grown on liquid cultures, have been chemically investigated and yielded alkaloids and quinones (Gill and Steglich, 1987), linear compounds (Birkinshaw et al., 1952), alkaloids (Cavill et al., 1953), pyrones (Ali et al., 1996) and terpenoids (Tai et al., 1995). Some of these compounds were isolated by bioassay-guided fractionation via monitoring their antibacterial and/or immunomodulatory activities.

*P. arcularius* has been previously chemically investigated (Fleck et al., 1996) yielding isodrimenediol, drimenediol and related sesquiterpenes. In the course of our studies on bioactive and new components from fungi (Cabrera and Seldes, 1997; Levy et al., 2000), new and known drimane terpenoids were isolated and identified from the fungal cultures of *Polyporus arcularius* and *P. ciliatus*. Different species of this genus were also examined, in order to correlate chemotaxonomic profiles with recent enzymatic and morphologic studies (Borges da Silva, 2001).

2. **Results and discussion**

Strains of *P. arcularius*, *P. ciliatus*, *P. philippinensis*, *P. guianensis* and *P. tenuiculus* were grown on a malt extract broth for two weeks and filtered. The filtrates were extracted with EtOAc to give crude extracts, which were analyzed by TLC, bioassays and by 1H NMR spectroscopy. The culture media extracts of *P. arcularius* and *P. ciliatus* showed major polar compounds, as evidenced by 1H NMR spectral analysis, with acidic characteristics as observed by TLC. The extracts corresponding to the other strains did not show any appreciable secondary metabolite production by 1H NMR and TLC. Based on these results, *P. arcularius* and *P. ciliatus* were cultured on a larger scale. Both extracts were subjected to HPLC yielding compounds 1 and 2 from *P. arcularius* and 3, 4 and 5 from *P. ciliatus*. 
The molecular formula of compound 1 was C_{21}H_{32}O_7, as determined by HRFABMS. Full structural assignments were made by interpretation of the 2D NMR spectra, including COSY, RCT, HETCOR, COLOC and NOESY. The 1H NMR spectrum showed three quaternary methyls at δ 0.81, 0.86 and 0.89, a broad singlet at 5.45 ppm characteristic of a double bond, a methyl group at δ 1.74 attached to a double bond and six signals between 2.5 and 4.1 ppm, indicative of the presence of heteroatoms in the molecule. The 13C NMR spectrum revealed the presence of a double bond with carbon chemical shifts at δ 123.2 and 134.3, two carbons attached to oxygen, at δ 70.3 (CH2) and δ 79.6 (CH), and three carbonyl carbons at δ 173.7, 174.2 and 175.0. COSY and RCT spectra allowed the identification of the partial structures—CH2–CH2–CH2–C–CH3, CH–CH2–CH–CH(CH3)–CH–CH2–O and O–CH–CH–CH2–. The structure of 1 was finally established by the detailed analysis of the COLOC spectrum. This experiment exhibited strong correlations between the methyl at δ 0.89 with the carbon at δ 33.6, the methyl at δ 0.86 with the carbons at δ 50.6, 42.8 and 22.2, and the methyl at δ 0.81 with the carbons at δ 55.4, 40.0 and 36.3, respectively. These correlations established a drimenol substructure as part of compound 1. The above 2D experiments thus permitted the identification of an isocitrinate moiety, which was connected to the drimenol portion via an ether linkage between C-11 and C-1’. All the spectroscopic data of the sesquiterpene were in accordance with those of drimenol (De Bernardi et al., 1980) supporting the relative stereochemistry of the sesquiterpenoid portion of the molecule.

In order to determine the absolute configuration of the molecule, a permethylated ester derivative 1a was prepared and compared by 1H NMR spectral analysis to the four diastereomeric cryptoporic acids recently synthesised (Tori et al., 2000). Compound 1a had 1H NMR signals corresponding to H-1’, H-2’ and 2H-3’ at δ 4.07, 3.45, 2.82 and 2.57 respectively, suggesting the absolute stereochemistry of either 1’S2’R or 1’R2’S. On the basis of the optical rotations of compound 1, drimenol and isocitrinic acid, and by comparison with cryptoporic acid H (Hirotani et al., 1991), the 1’R2’S stereochemistry was deduced for compound 1. From these data the structure of 1, named isocryptoporic acid H, was established as 3-carboxy-2-(2,5,5,8a-tetramethyl-1,4,4a,5,6,7,8,8a-octahydro-naphthalen-1-ylmethoxy)-pentanedioic acid. [The name isocryptoporic is proposed based on the isomeric relationship between compound 1 and cryptoporic acid H where a drimenol instead of an albacanol moiety is present in the molecule.]

Compound 2, C_{21}H_{32}O_8 by HRFABMS, had, as evidenced by 1H and 13C NMR spectral analyses, a similar pattern to compound 1, i.e. a double bond proton resonance at δ 5.44, the characteristic signals of the isocitric moiety at δ 4.08 (d, 4.6 Hz), 3.33 (m), 2.74 (dd, 17.0 and 9.8 Hz) and 2.54 (dd, 17.0 and 4.8 Hz), a vinyl methyl at δ 1.73, as well as a new signal at 3.18 (dd, 11.2 and 4.8 Hz). The 13C NMR spectrum exhibited also an additional methine carbon at δ 79.7 indicating the presence of a hydroxyl group in the molecule. Again the COSY spectrum allowed us to obtain partial structures which were connected by correlations observed in the COLOC experiment, identifying 3-hydroxydrimenol as the sesquiterpene portion of the molecule. COLOC and NOESY spectra showed correlations of H-11 protons with C-1’ and H-1’ respectively. Fig. 1 shows the most structurally relevant correlations observed. The absolute stereochemistry of 2 was assumed to be the same than compound 1. For the above mentioned reasons, the structure of 2 was established as 3-carboxy-2-(6-hydroxy-2,5,5,8a-tetramethyl-1,4,4a,5,6,7,8,8a-octahydro-naphthalen-1-ylmethoxy)-pentanedioic acid. Compound 2

Fig. 1. 2D NMR important correlations observed for compound 2.
was named isocryptoporic acid I by analogy with cryptoporic acid I.

Compound 3, C₈H₆O₄ by HREIMS, had a simple ¹H NMR spectrum with just three signals at δ 7.94 (1H), 6.49 (1H) and 4.40 (2H), whereas ¹³C NMR spectrum showed six carbon resonances, one carbonyl at δ 176.9, four aromatic carbons and a methylene group attached to oxygen. The chemical shifts were consistent with the presence of a furan ring bearing carboxyl and hydroxymethyl substituents. The small coupling (0.5 Hz) between the hydroxymethyl protons and the furan proton at 6.49 ppm suggested that they were on adjacent carbons. The furan protons lacked further coupling and were clearly not vicinal. To confirm this assumption compounds 3 were methylated to yield 3a and a NOESY experiment was carried out. A correlation between the O-methyl group and the signal at δ 7.55 confirmed the proposed structure for compound 3 as 5-hydroxymethylfuran-3-carboxylic acid. This compound has not been previously isolated as a natural product, but has been reported as a synthetic intermediate (Pevzner et al., 1999; Ionin, B.I., personal communication).

The ¹H and ¹³C NMR spectra of compound 5 resembled that of cryptoporic acid H (Hirotani et al., 1991) except for the appearance of a methyl ester group (¹H: δ 3.64 s, ¹³C: δ 52.5) and the upfield shift of one of the carbonyl groups in the ¹³C NMR spectrum. The upfield shifted carbonyl seemed to be 5’ by comparison of the ¹³C NMR spectra of 5 and cryptoporic H. COSY, TOCSY and ROESY spectra confirmed the proposed structure and the HMBC spectrum allowed us to determine the position of the additional methyl group. Fig. 2 shows the important correlations in the isocitric moiety observed in the HMBC experiment. These data and the similar optical rotation of compound 5 and cryptoporic acid H allowed us to establish the structure as 2”-O-methyl cryptoporic acid H.

Compound 4 was identified as cryptoporic acid H by comparison of spectroscopic data (¹H and ¹³C NMR spectra, FABMS) and optical rotation (Hirotani et al., 1991).

The extracts of P. philippinensis, P. guianensis and P. tenuiculus were analyzed by TLC and ¹H NMR and compared with the isolated compounds. In all cases compounds 1–5 were absent, even at trace levels. Remarkably, the above mentioned strains showed very poor productivity of secondary metabolites. In conclusion, only two of the studied strains produce drimane derivatives, P. arcularius and P. ciliatus. It is noteworthy that these strains belong to the same phenotypic group in phenograms based on morphological data, while the others belong to different groups (Borges da Silva, 2001). None of the isolated compounds exhibited antibiotic or antifungal activity in vitro.

Drimane sesquiterpenoids have been isolated from the basidiomycetes P. arcularius (Fleck at al., 1996) and Lactarius avidus (De Bernardi et al., 1980; Garlaschelli et al., 1994). Roseofomes subflexibilis (Nozoe et al., 1993), Haploporus odorus (Morita et al., 1995) and Cryptoporus volvatus (Hashimoto et al., 1987, 1989; Hirotani et al., 1991; Asakawa et al., 1992). However, in all the above cases the sesquiterpenoid portion of the drimane-isocitreric ethers is albicanol (Hirotani et al., 1991; Asakawa et al., 1992; Nozoe et al., 1993; Morita et al., 1995). This is the first report on the isolation of drimenol derivatives of this class, for which the name isocryptoporic acid is given.

3. Experimental

3.1. General

FTIR spectra were recorded on a Nicolet Magna-IR 550. The UV spectra were recorded on a Hewlett Packard 8451 A diode array spectrophotometer, whereas optical rotations employed a Perkin Elmer polarimeter 343. NMR spectra were acquired on a Bruker AM-500 instrument at 500.13 MHz for ¹H and at 125.13 MHz for ¹³C NMR, respectively. The NMR spectra of compound 5, however, were determined at the Instituto Nacional de Tecnologia Industrial (INTI, Bs As, Argentina) using a Bruker Advance DPX 400 instrument. FAB-MS were obtained on a ZAB-SEQ (BEqQ) instrument (VG Analytical, Manchester, UK), whereas HR–FABMS were recorded at the Washington University Resource for Bio-medical and Bio-organic Mass Spectrometry.

3.2. Fermentation

P. arcularius Batsch. ex Fr. (Cult. BAFC 109), P. philippinensis (Cult. BAFC 368), P. ciliatus (Cult. BAFC 2308), P. guianensis (Cult. BAFC 2793), P. tenuiculus (Cult. BAFC 162) were supplied by one of us [Dr. J.E. Wright] from the BAFC Culture Collection (FCEN-UBA, CONICET). An agar slant of each fungus was used to inoculate two 250 ml Erlenmeyer flasks containing 75 ml of malt extract medium composed of malt extract 30 g and peptone 5 g per liter. Fermentation was
carried out at 25 °C for 15 days under static conditions. 

_P. arcularius_ and _P. ciliatus_ were further cultivated seeding 2×4 l and 1×4 l Erlenmeyer flasks respectively containing 1 l of culture media with the above one week precultured media. Final pH was 4.4 (_P. arcularius_) and 5.0 (_P. ciliatus_).

### 3.3. Extraction and isolation

Fermentation broths of _P. arcularius_ and _P. ciliatus_ were filtered and the filtrates were partitioned with EtOAc. The extracts were subjected to HPLC (C18, 25×2 cm, MeOH–H2O 8:2, 6 ml/min, UV 215 nm, RI) yielding compounds 1 (51 mg) and 2 (37 mg) from _P. arcularius_ and 3, 4 (2.5 mg) and 5 (2.9 mg) from _P. ciliatus_. Compound 2 was purified by the same technique (C18, MeOH–H2O 7:3, 6 ml/min, UV 215 nm) and compound 3 was purified by prep. TLC on silica gel using EtOAc as elution solvent (RF 0.5) (17 mg). Compounds 1a and 3a were prepared by treatment of the corresponding compounds 1 and 3 with CH2N2 in Et2O.

### 3.4. Antibiotic assay

Antibiotic activity was determined by the agar diffusion method using 100 µg of sample/disk against _Bacillus subtilis_ ATCC 6663, _Staphylococcus aureus_ ATCC 25923 and _Escherichia coli_ ATCC 25922, _Candida albicans_ ATCC 18804 and _Cladosporium cucumerinum_ as test organisms. All compounds were inactive.

### 3.5. Compound 1

Oils. αD = +24° (MeOH; c 0.41). HRFAB+ MS (glycerol Na), m/z [M + 2Na-H]+, found 441.1854, calc. for C27H39O3Na2 441.1866. FAB+ MS (glycerol) m/z (rel. int.): 419 [M + Na]+ (30), 205 (35), UV (MeOH) λmax nm (log ε) 242 (3.17), 204 (3.48). FTIR (KBr) νmax cm⁻¹: 3422 (OH), 3222 (OH), 2924 (CH), 1737 (CO), 1719 (CO). For 1H and 13C NMR spectroscopic data for compounds 1, 2, 4 and 5 (CD3OD), see Table 1.

### 3.6. Compound 2

Oils. αD = +17° (MeOH; c 0.39). HRFAB+ MS (glycerol Na), m/z [M + 2Na-H]+, found 457.1808, calc. for C27H39O3Na2 457.1815. FAB+ MS (glycerol) m/z (rel. int.): 435 [M + Na]+ (45), 223 (85), 207 (100). UV (MeOH) λmax nm (log ε) 244 (3.34), 206 (3.37). FTIR (KBr) νmax cm⁻¹: 3451 (OH), 3210 (OH), 2945 (CH), 1740 br (CO), 1719 (CO). For 1H and 13C NMR spectroscopic data for compounds 1, 2 and 5 (CD3OD), see Table 2. For 1H NMR spectroscopic data for compounds 1, 2 and 5 (CD3OD), see Table 2.

### 3.7. Compound 3

Colorless needles (MeOH). Pf. 153–154 °C. HREIMS M++ m/z, found 142.0273, calc. for C6H6O4 142.0266. El MS (70 eV): 142 (M++), 100, 113 (79), 97 (18), 85 (38), 69 (95). UV (MeOH) λmax nm (log ε): 269 (3.78), 1740 br (CO), 1719 (CO). For 1H and 13C NMR spectra, see Tables 1 and 2.
3.8. Compound 5

Oil. $\alpha_D^0 = +32^\circ$ (CH$_3$OH; $c$ 0.23). HRFAB–MS (glycerol), $m/z$ [M–H]$^-$, found 409.2234, calc. for C$_{22}$H$_{33}$O$_7$ 409.2226. FAB–MS (glycerol) $m/z$ (rel. int.): 409 [M–H]$^-$ (100). UV (MeOH) $\lambda_{\text{max}}$ nm (log $\varepsilon$): 202 (3.89), 228 sh $\delta$ (3.33). FTIR (KBr) $v_{\text{max}}$ cm$^{-1}$ 3429 (OH), 3202 (NH), 2931 (CH), 1740 (CO), 1656 (CO), 1639 (CO), 1610 (CO), 1579 (s), 1534 (brt, 0.5 Hz, H-7), 1474 (C-5), 1410 (C-2), 1108 (C-4), 61.2 (C-7). Compound 3a. $^1$H NMR (CDCl$_3$): 7.55 (s, H-2), 6.52 (br $d$, 0.6 Hz, H-4), 4.48 (br $d$, 0.6 Hz, H-7), 3.76 (s, OCH$_3$).

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