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Pitholides A-D, Polyketides from a Marine Tunicate-Derived Culture of *Pithomyces* sp.

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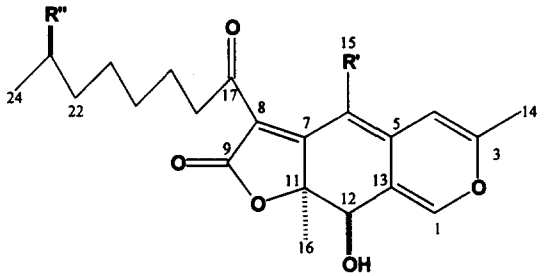
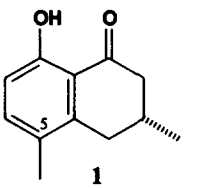
Abstract: Four new fungi pigments, pitholides A-D (**2-5**) were isolated from the salt water culture of the fungus *Pithomyces* sp. separated from the Indo-Pacific tunicate *Oxycorynia fascicularis*. Their structures include the core of Austdiol (**6**), an *Aspergillus* metabolite. © 1997 Elsevier Science Ltd.

There is a growing interest in exploring natural products of marine invertebrate-derived filamentous fungi.[1] To date, our attention in this area has been on sponges which have provided a library of fungal isolates and after salt water culture afforded terpenes, polyketides or nitrogen-containing compounds.[2] Locating organisms which produce nitrogenous metabolites is of increasing priority in our program.[2a] Particularly eye-catching was the 1994 report of andrimid obtained from the culture of the tunicate-derived bacterium *Pseudomonas fluorescens*.[3] In part, this stimulated the collection of a tunicate *Oxycorynia fascicularis* (coll. no. 96190) which was found to be a source of a fungus in the *Pithomyces* genus (coll. no. 969053). Significantly, cyclic depsipeptides such as pimaydolide and pithomycolide are obtained from *P. maydicus* and *P. chartarum*, respectively, and the latter is also a source of sporidesmin type diketopiperazines.[4] Given this interesting background, we launched a chemical investigation on the salt water culture of the tunicate-derived fungus and now report on the isolation of polyketides rather than nitrogen-containing compounds.



2 R'= CHO, R"= OH
3 R'= CHO, R"= H
4 R'= H, R"= OH
5 R'= H, R"= H



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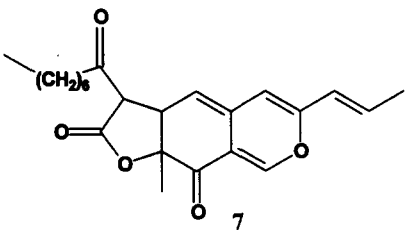

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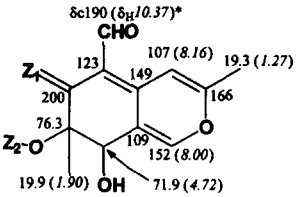
A marine media[2] was used to grow *Pithomyces* sp., and a total of 4 L (8 x 500 ml) was processed. Ethyl acetate extraction of the broth afforded an oil (610 mg) which was partitioned between 90% aqueous methanol and hexane followed by 50% aqueous methanol and methylene chloride. The hexane soluble fraction (60 mg) provided 30 mg of a pure compound, (*R*)-5-methylmellein (**1**), ($[\alpha]_D$ - 97.7° (*c* 0.65, $CHCl_3$), Lit.[5a] $[\alpha]_D$ -105° (*c* 0.5, $CHCl_3$)), which was previously isolated from *Aspergillus* sp., *Fusicoccum amygdali*, *Semecarpus* sp., and *Hypoxylon* sp.[5] This compound was toxic to brine shrimp at 0.1 mg/ml, but was not active as an *in vitro* cytotoxin.[6] The methylene chloride extract (FD) (610 mg) demonstrated equal cytoxicity in a soft agar-based

bioassay.[6] The zone of inhibition sizes observed are: [μg/disc: L1210/C38/H116] FD 600: 500/>800/700. The active FD fraction was chromatographed on Sephadex eluted with 1:1 CH_3OH-CH_2Cl_2 to yield 14 fractions. The activity was tracked into minor fractions 10 and 11, which are still under investigation. Alternatively, the major fractions 8 and 9 were purified by reversed phase HPLC using a CH_3OH-H_2O gradient to produce four new red pigments, pitholides A-D (**2**-**5**) (**2**: 16.3 mg, **3**: 15.7 mg, **4**: 3.9 mg, **5**: 4.6. mg). Even though these red oils were inactive in primary screens employing both brine shrimp and the soft agar cytotoxicity screen, the investigation was continued because new structures appeared to be in hand.

The first compound to be characterized was pitholide A (**2**). Its molecular formula $C_{22}H_{26}O_7$ was established from HRFABMS $[MH]^+$ *m/z* = 403.1756 (Δ 0.1 mmu of calcd.) and ESIMS $[M+Na]^+$ *m/z* = 425.1 data. The ^1H, ^{13}C and APT NMR data revealed three methyl, five methylene, five methine and nine quaternary carbons for a total of $C_{22}H_{24}$.[7] Thus, two hetero atom protons were evident and could be assigned to two OH groups at δ 68.3 (C12) and δ 67.1 (C23). Seven of the ten unsaturations were attributed to three carbonyls and four carbon-carbon double bonds identified by the ^{13}C NMR data, which meant three rings were present. Other important features included two vinyl protons as singlets, two tetrasubstituted carbon-carbon double bonds and a conjugated aldehyde. The connectivity relationships established from the HMBC data and the similarity of NMR shifts of **2** versus those of austdiol (**6**)[8] justified the assignment of two rings as substructure A. Next, an array, consisting of C17-C24, was identified by data as follows: an HMBC correlation (H18 to C17), several ^1H-^1H COSY correlations (H18 - H19 - H20 - H21 -H22 - H23 - $H_3$24), APT NMR data which showed C18-C22 are CH_2 groups, and the excellent agreement between the observed and calculated (ACD) ^{13}C shifts of C19 to C24. The two remaining unassigned carbon atoms, consisting of a carbonyl (C9) and sp^2 (C8), were envisioned to be connected and sandwiched between C17, C7 and the OC11 residues to form a five membered unsaturated lactone. This final substructure was consistent with the observation that no correlations were observed to C9 in the HMBC spectrum. Overall, the final proposed structure of **2** was parallel to that of another fungal metabolite monascorubrin (**7**)[9].

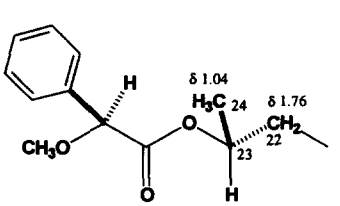


A: Z_1= Z_2= C

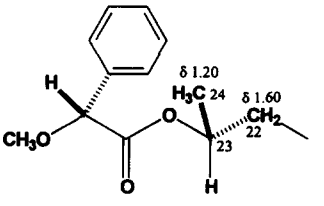
(+)-6: Z_1=O, Z_2= H

* NMR data for **6** see: Cole, R.J.; Cox, R.H. "Handbook of Toxic Fungal Metabolites" Academic Press, NY, 1981, 790

Attention was next turned towards the determination of absolute stereochemistry of C23 in **2**. This was accomplished by the preparation and ^1H NMR analysis of (*R*)- and (*S*)- MPA (2-methoxyphenylacetic acid) esters **2a** and **2b** (Figure 1). The $H_3$24 and $H_2$22 chemical shifts were **2a**: δ1.04 and δ1.76, **2b**: δ1.20 and δ1.60



(S)-MPA ester (**2a**)



(R)-MPA ester (**2b**)

Figure 1. The analysis of MPA esters of **2**

which, as shown in Figure 1, indicated that C23 was *R*. The relative *trans* stereochemistry of $CH_3$16 and OH12 was based on the NOESY correlation between $H_3$16 and H12, and the absence of a NOESY correlation between $H_3$16 and OH12 (in dioxane-d_8).

Having the structure of **2** as a model simplified the task of characterizing the remaining compounds. The HRFABMS peak $[MH]^+$ *m/z* = 387.1817 indicated the molecular formula of **3** as $C_{22}H_{26}O_6$ (Δ 0.9 mmu of cald.). The ^{13}C and APT data showed three methyl, six methylene, four methine and nine quaternary carbons totaling $C_{22}H_{25}$.[10] The only difference between pitholide B (**3**) and pitholide A (**2**) was the absence of the OH23 in **3**. In a similar fashion, the HRFABMS of pitholide D (**5**) indicated a molecular formula of $C_{21}H_{26}O_5$ (*m/z* = 359.1860 $[MH]^+$ Δ 0.2 mmu of cald.). The comparison of NMR spectra[11] of pitholide D (**5**) to those of **2** and **3** clearly showed that the aldehyde group in **2** and **3** was replaced by a singlet proton. Furthermore, H4 was shifted upfield to δ 6.36 because the substituent effect of the aldehyde group was absent. Additional HMBC correlations from H6 (δ 6.62) to C4, C11 and C13 further support the structure of **5**. Unfortunately, pitholide C (**4**) was isolated as a mixture even after repeated HPLC purification. However, it was easily identified by comparison of its ^1H NMR, ^{13}C NMR[12] and HMBC data with that of **5**. Overall, **4** was a C23 oxygenated derivative of **5**. Its molecular formula $C_{21}H_{26}O_6$ was established by the HRFABMS *m/z* = 375.1809 $[MH]^+$ (Δ 0.1 mmu of cald.) and by ESIMS *m/z* = 397.2 $(M+Na)^+$. Finally, the NOESY spectra indicated that **3** - **5** all have the same relative stereochemistry as **2** at positions C11 and C12, and the ^{13}C shifts along with biogenetic considerations suggested that C23 stereochemistry is identical for **2** and **4**.

The pitholides are an interesting structural class and are the first examples of this group to be isolated from the salt water culture of a marine-derived fungus. As noted above, there is a similarity in the structures of pitholide A (**2**) and monascorubrin (**7**) obtained from the fungus *Monascus purpureus*. Other similar pigments are also known from *M. purpureus* and include ankaflavin,[13] and monascorubramine.[14] Interestingly, these various fungal pigments have been shown to be responsible for the color in red rice which was used as a coloring additive in the preparation of certain foods and alcoholic beverages in some parts of Asia.[14] It must be assumed that compounds of the pitholide and monascorubrin family are benign and consistent with this observation is that neither **2** nor **3** were active in the brine shrimp assay at 0.2 mg/ml. However, this latter observation is in contrast to the brine shrimp assay toxicity noted for austdiol (**6**) plus literature data which shows **6** to be a gastrointestinal toxin.[8]

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7. Pitholide A (**2**): red oil, $[\alpha]_D$ -84° (*c* 0.59, CH_3OH), ^1H NMR (500 MHz, CD_3OD) δ (ppm) 8.02 (s, H1), 7.88 (s, H4), 4.51 (s, H12), 2.37 (s, 3H, H14), 9.78 (s, H15), 1.37 (s, 3H, H16), 2.85 (m, 2H, H18), 1.63 (m, 2H, H19), 1.41 (m, 6H, H20-22), 3.69 (m, H23), 1.13 (d, 3H, *J* = 6.5 Hz, H24). ^{13}C NMR (125 MHz, CD_3OD) δ (ppm) 153.4 (d, C1), 164.9 (s, C3), 106.3 (d, C4), 145.7 (s, C5), 107.0 (s, C6), 165.1 (s, C7), 118.2 (s, C8), 171.4 (s, C9), 83.5 (s, C11), 68.3 (d, C12), 120.6 (s, C13), 18.6 (q, C14), 186.3 (d, C15), 21.0 (q, C16), 199.2 (s, C17), 42.0 (t, C18), 23.4 (t, C19), 28.8 (t, C20), 25.2 (t, C21), 38.6 (t, C22), 67.1 (d, C23), 22.0 (q, C24).
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10. Pitholide B (**3**): red oil, $[\alpha]_D$ -158° (*c* 0.85, CH_3OH), ^1H NMR (500 MHz, CD_3OD) δ (ppm) H1 to H18 are identical to those of **2**, 1.62 (m, 2H, H19), 1.32 (m, 8H, H20-23), 0.89 (t, 3H, *J* = 6.5 Hz, H24). ^{13}C NMR (125 MHz, CD_3OD) δ (ppm) C1 to C20 are identical to those of **2**, 28.8 (t, C21), 31.4 (t, C22), 22.2 (t, C23), 13.0 (q, C24).
11. Pitholide D (**5**): red oil, $[\alpha]_D$ -248° (*c* 0.51, CH_3OH), ^1H NMR (500 MHz, CD_3OD) δ (ppm) 7.60 (s, H1), 6.36 (s, H4), 6.62 (s, H6), 4.45 (s, H12), 2.20 (s, 3H, H14), 1.39 (s, 3H, H16), 2.88 (m, 2H, H18), 1.60 (m, 2H, H19), 1.32 (m, 8H, H20-23), 0.89 (t, 3H, *J* = 6.5 Hz, H24). ^{13}C NMR (125 MHz, CD_3OD) δ (ppm) 149.7 (d, C1), 161.5 (s, C3), 109.3 (d, C4), 143.6 (s, C5), 101.0 (d, C6), 173.3 (s, C7), 110.7 (s, C8), 172.2 (s, C9), 83.1 (s, C11), 68.2 (d, C12), 120.6 (s, C13), 17.9 (q, C14), 23.2 (q, C16), 197.9 (s, C17), 40.8 (t, C18), 24.0 (t, C19), 29.0 (t, C20), 28.8 (t, C21), 31.5 (t, C22), 22.2 (t, C23), 13.0 (q, C24).
12. Pitholide C (**4**): red oil, compound **4** was isolated as a mixture. ^1H NMR (500 MHz, CD_3OD) δ (ppm) H1 to H18 are identical to those of **5**, 1.63 (m, 2H, H19), 1.35 (m, 4H, H20-21), 1.52 (m, H22), 3.70 (m, H23), 1.14 (d, 3H, *J* = 6.5 Hz, H24). ^{13}C NMR (125 MHz, CD_3OD) δ (ppm) C1 to C20 are identical to those of **5**, 25.3 (t, C21), 38.6 (t, C22), 67.1 (d, C23), 22.0 (q, C24).
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