Methanol Adduct of Puupehenone, a Biologically Active Derivative from the Marine Sponge *Hyrtios* Species

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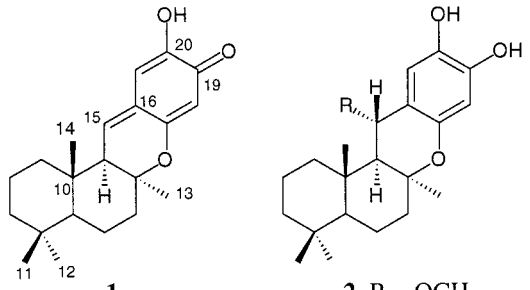
A methanol adduct of puupehenone (**1**), 15α-methoxypuupehenol (**2**), an artifact resulting from the action of MeOH on puupehenone, was isolated during purification of the CH_2Cl_2 extract of the New Caledonian marine sponge *Hyrtios* sp., as the major constituent. Its chemical structure was elucidated by 2D NMR experiments. Compound **2** displayed similar antimicrobial and antifungal activity as puupehenone and a lower cytotoxic activity toward KB cells with ED_{50} values of 6 and 0.5 μg/mL, respectively. Compound **2** was slightly more active against three strains of *Plasmodium falciparum* than puupehenone.

In the course of our biological research on puupehenone and its derivatives, we have reinvestigated the dichloromethane extract of the marine sponge *Hyrtios* sp., which is known to contain puupehenone[1] (**1**) and the red dimer dipuupehedione.[2] The ^{1}H NMR spectrum of the dichloromethane extract showed obviously that puupehenone was the major metabolite. Using a different purification procedure, a novel and major derivative was isolated, 15α-methoxypuupehenol (**2**), along with small amounts of puupehenone (**1**) and dipuupehedione. A complete chemical dataset of compound **2** and results of several biological assays are presented in comparison with puupehenone.



1 **2** R = OCH_3 **3** R = CN

Lyophilized specimens of the marine sponge *Hyrtios* sp. (800 g) were extracted with dichloromethane at room temperature during 2 days. This crude extract was fractionated by a Sephadex LH-20 gel-filtration ($CHCl_3$−MeOH 2:8). The first fractions afforded puupehenone (**1**) (0.1% dry wt) and dipuupehedione (0.01% dry wt). Compound **2** crystallized in MeOH from the following fractions as white needles, in large amounts (25% dry wt), and was identified by 1D and 2D studies and FABMS data.

The positive FABMS of compound **2** gave the M^+ molecular ion peak at *m*/*z* 360, corresponding to the molecular formula of $C_{22}H_{32}O_4$, which differs from that of puupehenone by an additional methoxyl group. The four methyl singlets at δ 0.62, 0.80, 0.89, and 1.18 ppm, observed in the ^{1}H NMR spectrum in $CDCl_3$, were reminiscent of the sesquiterpene moiety of puupehenone (**1**). However, the absence of the allylic proton, characteristic of puupehenone, and the presence of two new signals at δ 4.09 ppm (one proton) and 3.47 ppm (OMe) indicated a different substitution at position 15. An aromatic moiety was indicated by the presence of two singlet proton signals at 6.82 and 6.27 ppm. Full NMR data assignments for compound **2** were finally obtain by careful analysis of the HMQC and HMBC spectra in CD_3OD solution. Particularly, the HMBC correlations from the proton at δ 4.08 ppm to C-8, C-9, C-10, C-16, C-17, and C-21, unambiguously placed the methoxyl group at position 15. NOESY correlations between proton H-9, CH_3-12, and CH_3-13 indicated that they are all oriented on the same side, while a NOESY correlation between proton H-15 and CH_3-14 showed that they are oriented on the opposite side and confirmed the structure of 15α-methoxypuupehenol (**2**). Inspection of the ^{1}H NMR spectrum also revealed that coupling constant between H-9 (δ 1.55 ppm) and H-15 (δ 4.08 ppm) was of nearly 0 Hz, thereby indicating a dihedral angle close to 90°, similar to that observed for 15α-cyanopuupehenol (**3**).[3]

Compound **2**, the methanol adduct of puupehenone, was previously mentioned in the literature during isolation of the dimeric metabolite bispuupehenone.[4] It was also generated in cooling puupehenone to dry ice temperature,[5] upon warming puupehenone was regenerated quantitatively. In our study, fractionation of the crude extract in methanolic solution led to the formation of compound **2** and we regenerated puupehenone on a Si gel support. This allowed us to postulate that compound **2** is an artifact arising from addition of methanol to puupehenone. However, Zjawiony et al., while studying the chemistry of puupehenone and specifically the stereospecific 1,6-conjugate addition to its quinone−methide system, never obtained 15α-methoxypuupehenol by simple addition of methanol to puupehenone.[6] They succeeded in obtaining 15α-methoxypuupehenol diacetate, co-occurring with 15α-acetoxypuupehenol diacetate, from the reaction of puupehenone with magnesium methoxide in methanol, followed by acetylation of the crude mixture with acetic anhydride−pyridine.[6]

Compound **2** exhibited similar antimicrobial activity against *S. aureus* (7 mm of growth inhibitory zone at 1 μg/disk) and antifungal activity against *C. tropicalis* (9 mm of growth inhibitory zone at 50 μg/disk) as puupehenone (**1**). Compound **2** displayed a moderate cytotoxic activity toward KB cells, with an IC_{50} value of 6 μg/mL, while puupehenone (**1**) exhibited a higher cytotoxic activity, with an IC_{50} value of 0.5 μg/mL. However, a similar and very low toxicity, with LC_{50} values of approximately 20−30 μg/

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Table 1. *In Vitro* Antimalarial Activity (IC_{50} in μg/mL) against the Chloroquine-Susceptible F32 and the Chloroquine-Resistant FcB1 and PFB against *P. falciparum* Strains

	F32	FcB1	PFB
compound **2**	0.4	1.4	1.2
acetate of **2**	0.6	1.4	1.2
puupehenone (**1**)	0.6	2.1	1.5

mL against the brine shrimp lethality test, was observed. The antimalarial activity of compound **2** and its acetate was also investigated and compared with that of puupehenone against three different strains of *Plasmodium falciparum*. IC_{50} values are summarized in Table 1. From these data, it appears that compound **2** possessed the most interesting antimalarial activity toward all strains and that hydroxyl groups did not influence activity.

Experimental Section

General Experimental Procedures. MS were recorded on a Nermag 10−10 spectrometer (CI, NH_3), and HRFABMS (positive mode) was measured on a JEOL 700 spectrometer. IR and UV spectra were recorded on Nicolet (Impact 400D) and on Kontron-type Uvikon 930 spectrophotometers, respectively. ^{1}H and ^{13}C NMR spectra were obtained on a Bruker AC 300 spectrometer with standard pulse sequences operating at 300 and 75 MHz, respectively. The chemical shift values are in parts per million, and the coupling constants are in Hertz. The programs used for J_{mod}, HMQC, HMBC ($J = 7$ Hz) experiments were those furnished in the Bruker manual. Optical rotation was determined using a Perkin−Elmer 141 polarimeter with a sodium lamp operating at 589 nm in a 10-cm microcell. Si gel column chromatographies were carried out using Kieselgel 60 (230−400 mesh, E. Merck).

Collection and Isolation. Specimens of the sponge (2.5 kg, wet wt) *Hyrtios* sp. (family Thorectidae) were collected by scuba diving between −25 and −30 m depth from the East Coast of New Caledonia (Beautemps Beaupré area). Voucher specimens were deposited at ORSTOM under the reference R 1596 and identified by Prof. C. Lévi and J. Vacelet, France. Lyophilized specimens (800 g) were immersed in CH_2Cl_2 (total amount 4 L) at room temperature during 2 days. After filtration and concentration under vacuum, crude extract (8 g) was obtained, which was fractionated by a Sephadex LH-20 gel-filtration ($CHCl_3$−MeOH 2:8). The first fractions afforded puupehenone (**1**) (0.1% dry wt) and dipuupehedione (0.01% dry wt). Compound **2** crystallized in MeOH from the following fractions as white needles, in large amounts (25% dry wt), and was identified by 1D and 2D studies and FABMS data.

Compound 2: white needles; mp 124 °C; TLC (R_f 0.31); $[\alpha]^{20}_D$ −17° (*c* 0.8, $CHCl_3$); FT−IR (film) 3516, 3409, 2933, 1614, 1528, 1467, 1419, 1274, 1089 cm^{-1}; UV (MeOH) λ_{max} 206 (ϵ 23 725) and 298 nm (ϵ 5652);^{1}H NMR ($CDCl_3$, 300 MHz) δ 6.82 (1H, s, H-21), 6.27 (1H, s, H-18), 5.31 (1H, brs, OH), 5.10 (1H, brs, OH), 4.09 (1H, s, H-15), 3.47 (3H, s, OMe-22), 1.54 (1H, s, H-9), 1.18 (3H, s, Me-13), 0.89 (3H, s, Me-12), 0.80 (3H, s, Me-11), 0.62 (3H, s, Me-14); ^{1}H NMR (CD_3OD, 300 MHz) δ 6.69 (1H, s, H-21), 6.18 (1H, s, H-18), 4.08 (1H, s, H-15), 3.42 (3H, s, OMe-22), 2.01−1.60 (2H, m, H-7), 1.98−1.12 (2H, m, H-1), 1.64−1.58 (2H, m, H-2), 1.55 (1H, s, H-9), 1.52−1.40 (2H, m, H-6), 1.45−1.38 (2H, m, H-3), 1.01 (1H, m, H-5), 1.18 (3H, s, Me-13), 0.92 (3H, s, Me-12), 0.83 (3H, s, Me-11), 0.65 (3H, s, Me-14); ^{13}C NMR (CD_3OD, 75 MHz) δ 148.6 (C-17), 147.5 (C-19-OH), 140.1 (C-20-OH), 117.1 (C-21),115.2 (C-16), 104.6 (C-18), 75.8 (C-8), 75.1 (C-15), 56.4 (C-22), 56.2 (C-5), 54.8 (C-9), 42.9 (C-3), 41.9 (C-7), 40.9 (C-1), 38.1 (C-10), 34.3 (C-12), 34.2 (C-4), 27.9 (C-13), 22.5 (C-11), 19.5 (C-2 or C-6), 19.4 (C-6 or C-2), 15.1 (C-14); HR FABMS (positive) (*m*/*z* 360.2287, Δ mmu −1.4) 360, 329, 313, 247, 205, 177.

Acetylation of 2. Compound **2** (5 mg) with Ac_2O (0.5 mL) and pyridine (1 mL) was stirred overnight at room temperature. The mixture was poured onto ice and extracted with CH_2Cl_2. The organic phase was dried over Na_2SO_4 and evaporated to obtain the diacetate (3 mg): ^{1}H NMR ($CDCl_3$, 300 MHz) δ 7.10 (1 H, s), 6.60 (1 H, s), 4.08 (1 H, s), 3.42 (3H, s, OMe), 2.22 (6H, s, OAc), 1.19 (3H, s), 0.89 (3H, s), 0.79 (3H, s), 0.62 (3H, s).

Antibacterial and Antifungal Assays. Antibacterial and antifungal activities were tested against a Gram positive *S. aureus* and a Gram negative *E. coli* and the fungus *C. tropicalis* by the paper disk method. Compounds were dissolved in MeOH. Growth inhibitory zones were recorded after 24 h for bacterial cultures and 48 h for fungal cultures.

Cytotoxicity Assay. The cytotoxicity experiments against human nasopharyngeal carcinoma cell lines (KB) were performed according to the methodology previously reported.[7] KB cells were counted by using neutral red as dye, and absorbances were measured at 540 nm in a microplate reader (Ceres 900-Bio-tek Instruments).

Brine Shrimp Bioassay. A convenient bioassay using newly hatched nauplii of the brine shrimp *Artemia salina* was performed following the methodology described by Meyer et al.[8] The procedure determines LC_{50} values in μg/mL of compounds displaying toxicity.

Antimalarial Assay. The experiments were performed with the chloroquine-resistant FcB1/Columbia, chloroquine-resistant PFB/Brazil, and chloroquine-susceptible F32/Tanzania strains. All the drug concentrations were tested in triplicate for each experiments. The detailed methodology has been described by Desjardins et al.[9]

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