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Studies on puupehenone-metabolites of a *Dysidea* sp.: structure and biological activity

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Abstract—Puupehenone (**1**) and a series of its congeners (**2**–**6**) have been isolated from a *Dysidea* sponge. The unprecedented 20-acetoxy-haterumadienone (**2**) exhibiting a five-membered contracted ring, has been characterized. In addition, stereochemical assignment of two previously reported acetone adducts of puupehenone (**5** and **6**) has been made. Finally, the inhibition of mitochondrial respiratory chain as well as antibacterial and antifungal activities of all compounds has been evaluated.

1. Introduction

Sesquiterpene-substituted quinones and related molecules constitute an important class of bioactive marine natural products.[1] Members of this group include tetracyclic puupehenone (**1**) and its numerous derivatives displaying large variations in their conjugation patterns and oxygen functionalization.[2] First isolated by Scheuer and co-workers from the sponge tentatively identified as *Chondrosia chucalla*,[3] puupehenone (**1**) has so far been isolated from different sponges mainly of orders Verongida and Dictyoceratida.[4] Puupehenone (**1**) exhibits a highly reactive quinone-methide moiety that facilitates the formation of C/D ring analogues, many of which are the products of 1,6-conjugate addition reactions by nucleophilic attack at C-15 of the parent compound **1**.[5] Puupehenone and related molecules have been the subject of sustained interest in the last years also due to their wide range of biological properties as antitumor, antiviral, antimalarial, antibiotic, immuno-modular and, most remarkably, antituberculosis activities displayed in vitro by **1** and its congeners.[4–6] These studies have resulted in the identification of several natural puupehenone-derived metabolites and also in the enantiospecific synthesis of the parent compound **1**.[7]

In the course of our chemical investigation of marine benthic invertebrates from Indo-Pacific littorals, we have examined the secondary metabolite pattern of a *Dysidea* sp. sponge from South China Sea. In this paper we report the structural elucidation of an unprecedented ring-contracted congener of puupehenone, 20-acetoxy-haterumadienone (**2**), co-occurring with the main metabolite, puupehenone (**1**), and other related molecules, the dimer bis-puupehenone (**3**), previously found in the Pacific sponge *Hyrtios eubamma*,[8] haterumadienone (**4**), and the acetone adducts **5** and **6**, very recently isolated from an Okinawan *Dysidea* sp. sponge.[9] The stereochemical assignment of the two epimers **5** and **6**, not reported previously, is also described here. Finally, the results of screening biological properties, including mitochondrial respiratory chain inhibition, of all compounds are reported.

2. Results and discussion

The frozen sponge was extracted with acetone and the acetone extract was partitioned between diethyl ether and water. The Et_2O phase was concentrated under reduced pressure affording a crude residue (1 g), which was analyzed by TLC chromatography, using different eluent systems. The secondary metabolite pattern was found to be dominated by the presence of a main UV-sensitive spot at R_f 0.7 (light petroleum ether/diethyl ether, 1:1) along with a series of minor components. The extract was fractionated by LH-20 Sephadex chromatography to give two main fractions containing sesquiterpene-quinone compounds. Both fractions were

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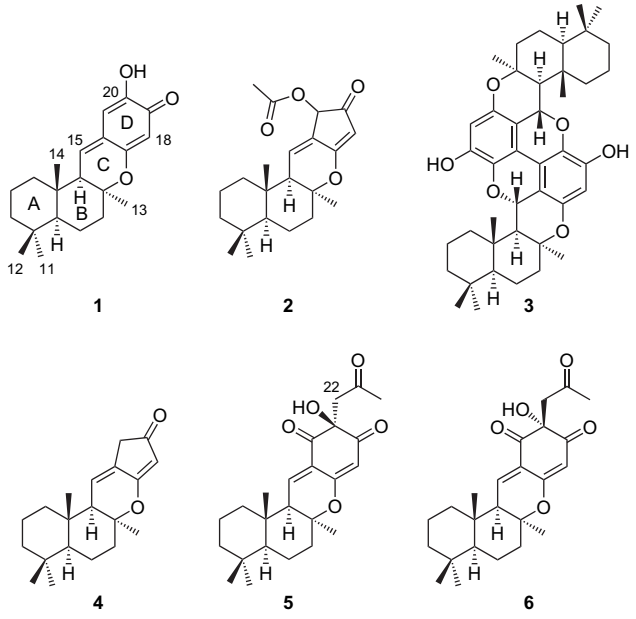
submitted to silica gel column purification to give, in order of polarity: compound **3** (40 mg), **1** (300 mg), and two more polar mixtures A and B at R_f 0.4 and R_f 0.2 (light petroleum ether/diethyl ether, 1:1), which were further purified by reverse-phase HPLC (MeOH/H_2O, 85:15) to obtain compounds **2** (1.0 mg) and **4** (1.6 mg) from A, and **5** (2.3 mg) and **6** (2.1 mg) from B. Puupehenone (**1**),[3,4] bis-puupehenone (**3**),[8] haterumadienone (**4**),[9,10] and the acetone adducts **5** and **6**[9] of a putative trione **7**, not detected in the extract, were identified by comparing their spectral data (^{1}H and ^{13}C NMR, MS, and $[\alpha]_D$ value) with the literature.



Compound **2** showed a molecular formula of $C_{22}H_{30}O_4$ as deduced by the sodiated-molecular peak at *m/z* 381.2031 in the HRESIMS spectrum. The ^{1}H and ^{13}C NMR spectra exhibited signals that strongly resembled those of haterumadienone (**4**)[10] (Table 1). The proton and carbon signals of the terpene rings A and B were almost identical to those of the corresponding part of haterumadienone, whereas differences were observed in the five-membered ring C pattern. In particular, in the ^{1}H NMR spectrum of **2**, a 3H singlet at δ 2.19, attributable to an acetyl group, and a deshielded 1H singlet at δ 5.67 (H-20) were observed in the place of the AB system resonating at δ 2.97 and δ 3.05 (H_2-20) of compound **4**, suggesting that **2** differed from **4** only in the presence of an acetoxy group at C-20. Analysis of 1D and 2D NMR spectra of compound **2** led to the assignment of all carbon and proton resonances (Table 1) and also confirmed the proposed structure, including the relative trans–*anti*–cis junction stereochemistry of A-, B- and C- rings, the same as in puupuhenone (**1**). The stereochemistry at C-20 of compound **2** remained undetermined.

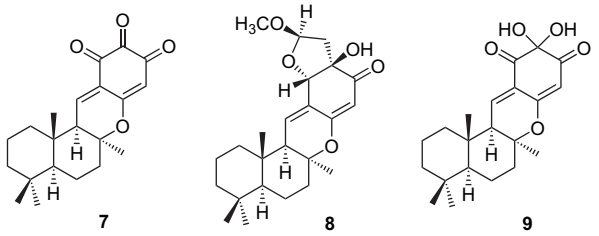
Spectral data (see Section 4) of compounds **5** and **6** were identical with those reported for the two C-20 epimers isolated from the extract of an Okinawan *Dysidea* sponge and suggested these compounds to be the acetone adducts of the putative trione **7**.[9] In order to establish the stereochemistry at C-20, not assigned in the previous paper,[9] a series of NOE difference experiments were carried out. Diagnostic steric effects were observed between H-22a (δ 2.97) and H_3-13 (δ 1.28) in compound **5**, and between H-22a (δ 2.86) and H_3-14 (δ 0.91) in compound **6**, indicating that the acetonyl residue at C-20 was α-oriented in **5** and β-oriented in **6**. This stereochemical assignment was also in agreement

Table 1. ^{1}H and ^{13}C NMR data[a] of compounds **2** and **4**

Position	**2**			**4**		
	δ_C, m[b]	δ_H	m, *J* (Hz)	δ_C, m[b]	δ_H	m, *J* (Hz)
1		1.60	m		1.68	m
	39.7, CH_2	1.12	dt (3.0, 12.7)	39.7, CH_2	1.11	dt (3.5, 13.1)
2	18.3,[c] CH_2	1.45	m	18.4,[c] CH_2	1.45	m
3		1.47	m		1.46	m
	41.6, CH_2	1.22	m	41.7, CH_2	1.22	m
4	33.1, qC			33.2, qC		
5	54.0, CH	0.97	dd (11.7, 2.1)	54.1, CH	0.97	dd (11.0, 2.6)
6	18.1,[c] CH_2	1.54	m	18.1,[c] CH_2	1.58	m
7		2.25	dt (2.9, 14.7)		2.19	dt
	39.2, CH_2	1.66	td (5.2, 14.0)	39.4, CH_2	1.65	m
8	80.7, qC			81.0, qC		
9	53.7, CH	1.97	d (6.6)	54.1, CH	1.92	d (6.5)
10	40.2, qC			40.0, qC		
11	33.7, CH_3	0.92	s	33.7, CH_3	0.92	s
12	21.9, CH_3	0.85	s	21.9, CH_3	0.85	s
13	28.8, CH_3	1.28	s	28.8, CH_3	1.27	s
14	14.5, CH_3	0.80	s	14.7, CH_3	0.82	s
15	124.6, CH	6.16	d (6.6)	122.3, CH	5.98	d (6.5)
16	133.2, qC			132.3, qC		
17	177.0, qC			180.0, qC		
18	107.1, CH	5.45	s	109.7, CH	5.45	br d (1.3)
19	197.0, qC			205.0, qC		
20	70.7, CH	5.67	s	37.7, CH	2.97	d (20.0)
					3.05	d (20.0)
21	171.0, qC					
22	21.0, CH_3	2.19	s			

[a] $CDCl_3$, Bruker Avance-400 spectrometer. Assignments aided by COSY, HSQC, HMBC.
[b] Multiplicity deduced by DEPT.
[c] Interchangeable.

with the stereochemistry reported for related compounds such as hyrtenone (**8**),[4i] exhibiting an additional α-oriented 20,21 cis-fused ring. Comparison of ^{1}H NMR chemical shift values of the two angular methyl groups, H_3-13 and H_3-14, in the two epimers **5** and **6** with those of hyrtenone (**8**) showed similar resonances for **5** [δ 1.28 (H_3-13), δ 0.81 (H_3-14)] and **8** [δ 1.25 (H_3-13), δ 0.80 (H_3-14)], whereas these values were significantly different in **6** [δ 1.18 (H_3-13), δ 0.91 (H_3-14)]. The discovery of compounds **5** and **6** should indicate the presence, in the sponge metabolite pattern, of the putative trione **7** or its hydrate form **9**. However, we were unable to detect such probable precursors.



All compounds (**1**–**6**) were assayed as inhibitors of integrated electron transfer chain (NADH oxidase activity) in beef heart submitochondrial particles (SMP). All of them showed a range of inhibitory potency similar to other interesting inhibitors of the mammalian mitochondrial respiratory chain such as benzopyranes or stolonoxides.[12,13] Compound **1** was found to be the most potent inhibitor of the integrated electron transfer chain (NADH oxidase activity) with IC_{50} values of 1.3±0.1 μM whereas full inhibition of rotenone-sensitive NADH oxidase activity was achieved at approximately 2 μM. Compounds **2** and **3** showed IC_{50} values between 17 and 34 times higher than **1** (Table 2), whereas for compounds **4**, **5**, and **6** IC_{50} values were observed below 10 μM. It is noteworthy that the placement of the acetoxy group at C-20 in compound **2** has a significant effect on activity, by decreasing the cytotoxic activity 5 times with respect to the related compound **4**.

All compounds **1**–**6** were tested for antifungal and antibacterial activities. No growth inhibition was exhibited on *E. coli* by the studied metabolites. Compound **1** showed moderate activity against *Candida albicans* and *Staphylococcus aureus*, whereas compounds **5** and **6** were the most active, with an MIC of 32 and 64 μg/mL against *C. albicans* and an MIC of 8 and 16 μg/mL against *S. aureus*, respectively.

Table 2. Inhibitory potency of compounds **1**–**6** against NADH oxidase

Inhibitors	IC_{50} (μM)
1	1.28±0.09
2	22.33±3.03
3	44.25±9.54
4	4.71±0.20
5	6.74±0.45
6	7.33±0.45

3. Conclusion

In conclusion, this study has added a novel member to the interesting class of puupehenone-metabolites and has clarified the stereochemistry of two previously reported molecules. Some interesting biological activities, including the inhibition of the mitochondrial respiratory chain as well as antibacterial and antifungal properties, have been evidenced for some of the isolated compounds.

4. Experimental section

4.1. General experimental procedures

Silica gel chromatography was performed using pre-coated Merck F_{254} plates and Merck Kieselgel 60 powder (Darmstadt, Germany). Sephadex LH-20 for molecular exclusion chromatography was purchased from Pharmacia (Uppsala, Sweden). HPLC purification was carried out on a Shimadzu SCL-10A liquid chromatograph equipped with a Shimadzu UV SPD-10A detector. Optical rotations were measured on a Jasco DIP 370 digital polarimeter. IR spectra were recorded on a Biorad FTS 155 FTIR spectrophotometer. UV measurements were carried out on Agilent 8453E UV–visible apparatus.

NMR experiments were recorded at ICB NMR Service. 1D and 2D NMR spectra were acquired in $CDCl_3$ (δ values are reported referred to C*H*Cl_3 at 7.26 ppm) on a Bruker Avance-400 spectrometer operating at 400 MHz, using an inverse probe fitted with a gradient along the *z*-axis, and on a Bruker DRX-600 spectrometer operating at 600 MHz, using an inverse TCI CryoProbe fitted with a gradient along the *z*-axis. ^{13}C NMR were recorded on a Bruker DPX-300 spectrometer operating at 300 MHz (δ values are reported to $CDCl_3$, 77.0 ppm) using a dual probe.

Low and high resolution ESIMS were performed on a Micromass Q-TOF Micro™ coupled with an HPLC Waters Alliance 2695. The instrument was calibrated by using a PEG mixture from 200 to 1000 MW (resolution specification 5000 FWHM, deviation <5 ppm rms in the presence of a known lock mass).

4.2. Biological material

A sample of the sponge *Dysidea* sp. was collected by SCUBA diving at a depth of 20 m along the coast of Hainan in South China Sea, during January 2002. Biological material was immediately frozen and stored at −20 °C till the extraction. The taxonomic identification has been made by one of us (R.v.S.). A voucher specimen is deposited at Zoological Museum, University of Amsterdam (code ZMA-POR19068). The species is a rose-purple lobate sponge with a densely conulose surface, heavily overgrown with filamentous red algae and encrusted with calcareous sediment. Conules were approximately 1 mm high and 2 mm apart. Oscules were variable in size, up to 3 mm in diameter, flush with the surface. Consistency was coarsely compressible. The interior was heavily sedimented with barely distinguishable debris-filled main fibers of 0.3–2 mm diameter interconnected irregularly with thinner debris-filled secondary fibers. Agglutinating spongin was barely detectable. The heavily sedimented interior distinguishes this species from all known *Dysidea* species of the West Pacific area.

4.3. Isolation procedure

The frozen sponge (64 g dry weight) was extracted with acetone (3×600 mL, 10 min in ultrasonic bath). The acetone extract was evaporated under vacuum and the resulting aqueous phase was extracted with Et_2O (3×300 mL). After evaporation of the solvent the organic layer afforded a crude gummy residue (1 g), which was subjected to an LH-20 Sephadex column chromatography by eluting with $CHCl_3$/MeOH, 1:1. Four main fractions were obtained: fr. I (192 mg), fr. II (120 mg), fr. III (508 mg), and fr. IV (20 mg), two of which (fractions II and III) contained sesquiterpene-quinone compounds. Fraction II was submitted to a silica gel column purification (light petroleum ether/diethyl ether gradient) to give bis-puupehenone (**3**, 40 mg) and two more polar fractions (fr. A and B) that were further purified on HPLC reverse-phase (MeOH/H_2O, 85:15, Kromasil RP-18 analytical column, flow rate 1 mL/min). Pure minor compounds **2** (1.0 mg) and **4** (1.6 mg) were obtained from fraction A, as well as **5** (2.3 mg) and **6** (2.1 mg) were recovered from fraction B. Fraction III (508 mg) was purified on a silica gel column eluting with a light petroleum ether/diethyl ether gradient to give puupehenone (**1**, 300 mg).

Compounds **1** and **3–6** were identified by comparing their spectral data (^1H and ^{13}C NMR, MS, and $[\alpha]_D$ value) with literature data.

Compound ***2***: colorless oil; $[\alpha]_D^{25}$ −21.2 (*c* 0.10, CCl_4); UV (MeOH) λ_{max} (log ε) 214 (8.91), 280 (9.47); IR (liquid film) ν_{max}: 2955, 2923, 2867, 2124, 2090, 1736, 1646, 1456, 895 cm^{-1}; ^1H and ^{13}C NMR: see Table 1; HRESIMS: found 381.2031 (381.2042 calculated for $C_{22}H_{30}O_4Na$).

Compound ***5***: $[\alpha]_D^{25}$ −28.9 (*c* 0.11, $CHCl_3$);[11] ^1H NMR ($CDCl_3$, 600 MHz) δ 1.80 (1H, m, H-1eq), 1.30 (1H, m, H-1ax), 1.50 (2H, m, H_2-2), 1.44 (1H, m, H-3a), 1.23 (1H, m, H-3b), 1.02 (1H, br d, 10 Hz, H-5), 1.61 (1H, m, H-6a), 1.43 (1H, m, H-6b), 2.26 (1H, m, H-7eq), 1.68 (1H, m, H-7ax), 2.21 (1H, d, 6 Hz, H-9), 0.94 (3H, s, H_3-11), 0.85 (3H, s, H_3-12), 1.28 (3H, s, H_3-13), 0.81 (3H, s, H_3-14), 7.44 (1H, d, 6 Hz, H-15), 5.70 (1H, s, H-18), 2.97 (1H, d, 14 Hz, H-22a), 3.05 (1H, d, 14 Hz, H-22b), 2.20 (3H, s, H_3-24).

Compound ***6***: $[\alpha]_D^{25}$ −40.7 (*c* 0.11, $CHCl_3$);[11] ^1H NMR ($CDCl_3$, 600 MHz) δ 1.78 (1H, m, H-1eq), 1.25 (1H, m, H-1ax), 1.50 (2H, m, H_2-2), 1.44 (1H, m, H-3a), 1.23 (1H, m, H-3b), 1.02 (1H, br d, 10 Hz, H-5), 1.67 (1H, m, H-6a), 1.52 (1H, m, H-6b), 2.28 (1H, m, H-7eq), 1.65 (1H, m, H-7ax), 2.20 (1H, d, 6 Hz, H-9), 0.93 (3H, s, H_3-11), 0.86 (3H, s, H_3-12), 1.18 (3H, s, H_3-13), 0.91 (3H, s, H_3-14), 7.35 (1H, d, 6 Hz, H-15), 5.70 (1H, s, H-18), 2.86 (1H, d, 14 Hz, H-22a), 3.02 (1H, d, 14 Hz, H-22b), 2.23 (3H, s, H_3-24).

4.4. Mitochondrial respiratory chain assay

The inhibitory potency of the compounds was assayed by using SMP from beef heart.[14] SMP were diluted to 0.5 mg/mL in 250 mM sucrose, 10 mM Tris–HCl buffer, pH 7.4, and treated with 300 μM NADH in order to activate complex I before starting the experiments. The enzymatic activity was assayed at 22 °C in 50 mM potassium phosphate buffer, pH 7.4, 1 mM EDTA with SMP diluted to 6 μg/mL. Stock solutions (15 mM in absolute EtOH) of **1–6** were prepared and kept in the dark at −20 °C. Each compound was added to the diluted SMP preparation and incubated, during 5 min, in ice. NADH oxidase activity was measured as the aerobic oxidation of 75 μM NADH. Reaction rates were calculated from the linear decrease of NADH concentration (λ 340 nm, ε 6.22 mM^{-1} cm^{-1}) in an end-window photomultiplier spectrophotometer ATI-Unicam UV4-500. For each compound, three experiments were carried out.

4.5. Antifungal and antibacterial assays

Antifungal assay was performed by the broth macrodilution method following the guidelines of the National Committee for Clinical Laboratory Standards (NCCLS) document M27-P.[15] The medium used to prepare the 10× drug dilutions and the inoculum suspension was liquid RPMI 1640 with L-glutamine (Sigma Aldrich), 0.165 M morpholinopropanesulfonic acid (MOPS), and 2% glucose (pH 7.0).[16,17] The yeast suspension was adjusted with the aid of a spectrophotometer to a cell density of 0.5McFarland (2×10^8 CFU/mL) standard at 530 nm and diluted to 1:4000 (50,000 CFU/mL) in RPMI 1640 broth medium. The yeast inoculum (0.9 mL) was added to each test tube that contained 0.1 mL of ten 2-fold dilutions (256–0.05 μg/mL final) of each compound. Broth macrodilution MICs were determined after 48 h of incubation at 35 °C. MIC was defined as the lowest concentration of the compound that completely inhibited the growth of the test organism. The antibacterial assay was performed by using the same method as the antifungal test, only differing in the assay medium (Luria Bertani medium: 10 g/L Bactotryptone, 5 g/L Bactoyeast, and 10 g/L NaCl, pH 7.5) and in the incubation temperature (37 °C for 24 h).

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