Meroterpenes from the Ascidian *Aplidium aff. densum*

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Received March 30, 2005

Chemical investigation of the ascidian *Aplidium aff. densum* collected at Masirah Island, Oman, has resulted in the isolation of five meroterpenes: two new ones, methoxyconidiol (**1**) and didehydroconicol (**2**), and three related, known compounds, **3**−**5**. The structures of **1** and **2** were determined by a combination of mass spectrometry and one- and two-dimensional high-field NMR techniques. Their biological activities against bacteria and human lymphoblastic cell lines were evaluated.

Many natural products of mixed biosynthesis, such as prenylated quinone/hydroquinone derivatives, have been reported from both terrestrial and marine sources. These are mostly hydroquinones with a terpenoid portion ranging in size from one to nine isoprene units. In marine organisms prenylated quinone/hydroquinone derivatives are mainly metabolites of brown algae, but other sources include green algae, sponges, ascidians, and octocorals. Ascidians of the genus *Aplidium*, *A. californicum*,[1a−c] *A. constellatum*,[2] *A. multiplicatum*,[3] *A. antillense*,[4] *A. solidum*,[5] or *A. savignyi*,[6] have been previously reported to yield geranylhydroquinone and diprenyl-quinone, -hydroquinone, -chromene, or -chromane derivatives. About 20 cyclofarnesylated hydroquinones or quinones, longithorones,[7a−c] and longithorols[8a,b] have been described from *Aplidium longithorax*. From *Synoicum castellatum*, a species closely allied to the genus *Aplidium*, Carroll et al.[9] reported the isolation of geranylhydroquinone, cordiachromene A (**3**), and compound **4**, which they named as a tetrahydrocannabinol derivative. New linear and cyclized diprenylhydroquinones, such as conidione (**5**), conidiol, and conitriol were also isolated from the Mediterranean tunicates, *Aplidium* sp.[10] and *A. conicum*.[11] Several of these marine metabolites of mixed terpene-shikimate biosynthetic pathways displayed interesting cytotoxic, antiinflammatory, antifungal, and anti-HIV activities.

We report the isolation and structure elucidation of two new meroterpenes, methoxyconidiol (**1**) and didehydroconicol (**2**), from *Aplidium aff. densum* along with the already reported compounds **3**, **4**, and **5**. The five compounds were evaluated for antibacterial and antiproliferative activities.

A sample of *Aplidium aff. densum*, collected near Masirah Island (Oman), was cleaned rapidly and then stored in ethanol until workup. The biological material was extracted with MeOH and CH_2Cl_2. After concentration, the crude extract was partitioned between water and ethyl acetate. The organic matter was subjected to silica gel column chromatography followed by reverse-phase HPLC of the fractions exhibiting blue coloration with Gibbs reagent,[12] suggesting the presence of phenolic compounds. The known compounds cordiachromene A (**3**), conidione (**5**), and compound **4**, which we named epiconicol, and new compounds **1** and **2** were isolated (Figure 1).

Methoxyconidiol (**1**) was obtained as a yellow oil. The molecular formula was established as $C_{17}H_{24}O_3$ by HRE-

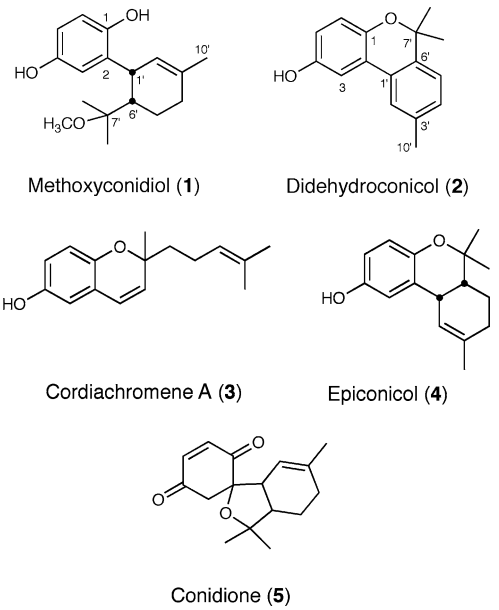

Figure 1. Structures of the five meroterpenes isolated from *Aplidium aff. densum.*

IMS, indicating six degrees of unsaturation. Ions resulting from the losses of OCH_3 (*m/z* 244) and H_2O (*m/z* 225) were observed together with the ion at *m/z* 161, which is indicative of the formation of a chromene moiety by an intramolecular condensation charasteristic of prenylated hydroquinones.[6] The ^{1}H NMR signals (Table 1) at δ 6.73 (H-6, d, J = 8.4 Hz), 6.61 (H-3, d, J = 3.0 Hz), and 6.57 (H-5, dd, J = 8.4 and 3.0 Hz) indicated the presence of the 1,2,4-trisubstituted aromatic ring. These protons were attached to aromatic carbon atoms at δ 118.2 (C-6), 118.8 (C-3), and 114.3 (C-5) all determined from interpretation of the HSQC data (Table 1). Long-range couplings observed in the HMBC spectrum of **1** between quaternary carbon signals at δ 149.4 and 130.5 (C-2) and aromatic protons H-3 and H-6, plus the presence of another signal at δ 148.4, suggested that this aromatic moiety was a 2-substituted *para*-hydroquinone ring. In addition, the two phenolic exchangeable protons were observed as sharp singlets (δ 8.45 and 4.25) in the ^{1}H NMR spectrum of **1**. The IR (3319, 1608 cm^{-1}) and UV (228, 274, 296 nm) spectra confirmed the presence of the hydroquinone moiety. The remaining 11 resonances, in the ^{13}C NMR spectrum, gave evidence for the three apliphatic methyl groups, one methoxy group, two methylene and three methine groups, and two quartenary carbon atoms. All of the foregoing data were

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10.1021/np050110p CCC: $30.25
Published on Web 09/08/2005

Table 1. ^{1}H and ^{13}C NMR Assignments of Methoxyconidiol (**1**)

atom no.	δ_C	δ_H (mult., *J*/Hz)	HMBC[a]
1	149.4		H-3, H-6, (C-1) OH
2	130.5		H-6
3	118.8	6.61 (d, 8.4)	
4	148.4		
5	114.3	6.57 (dd, 8.4; 3.0)	H-6
6	118.2	6.73 (d, 8.4)	(C-1) OH
1′	34.4	3.75 (dd, 5.5; 4.6)	
2′	125.6	5.32 (dq, 5.5; 1.5)	H-10′
3′	133.8		H-10′
4′	31.8	2.13 (m)	H-10′
5′	20.6	1.39 (m)	
		1.64 (m)	
6′	47.4	2.08 (ddd, 12.7; 4.6; 1.9)	H-8′, H-9′
7′	79.5		H-8′, H-9′, H (OCH_3)
8′	25.7	1.21 (s)	H-9′
9′	19.1	0.54 (s)	H-8′
10′	23.3	1.71 (bs)	
OCH_3	49.1	3.33 (s)	
(C-1) OH		8.45 (s)	
(C-4) OH		4.25 (s)	

[a] Proton showing long-range correlation to indicated carbon.

consistent with a diprenyl moiety organized in one ring with one double bond. Analysis of the ^{1}H NMR signals and correlations observed from the DQF-COSY H−H spectrum led us to build the following spin system: a methyl group (H-10′, bs) at δ 1.71, an ethylenic proton (H-2′) at δ 5.32, two methine groups H-1′ and H-6′ at δ 3.75 and 2.08, and the four methylene protons H-4′ and H-5′ at respectively 2.13, 1.39, and 1.64 ppm. Long-range correlations observed between C-4′ and H-10′ supported closing the 3,4-disubstituted-1-methylcyclohexene ring. Long-range couplings between the signal of the quaternary carbon at δ 79.5 (C-7′), the two singlets (3H) at δ 1.21 (H-8′) and 0.54 (H-9′), and the deshielded one at δ 3.33 (OCH_3) indicated the presence of a methoxyisopropyl unit linked to C-6′. To agree with the molecular formula, C-1′ of the second ring was attached to the quaternary carbon (C-2) of the hydroquinone ring. Long-range couplings between the most deshielded signal (δ 8.45) and carbon signals at 149.4 and 118.2 ppm allowed the complete assignment of compound **1**. The high-field chemical shift (δ 0.54) of the methyl group H-9′, unusual for a methyl group attached to an oxygen-bearing methine, indicated an interaction between the methyl group and the Π-cloud of the hydroquinone ring. The relative configuration of the two stereocenters of methoxyconidiol was assumed to be the same as in conitriol,[10] with a *cis* orientation of the substituents about the cyclohexene ring, due to very similar vicinal ^{1}H−^{1}H coupling constants and ^{13}C and ^{1}H chemical shifts observed between the two compounds. Furthermore the $J_{H1'-H6'}$ of 4.6 Hz and the NOEs observed between H-1′ and H-6′ and between H-1′ and the methoxy group are consistent with the *cis* relationship where the hydroquinone ring was in an axial position and the methoxyisopropyl group in an equatorial orientation. Such a conformation accounted for the shelding effect of the hydroquinone ring on the methyl group H-9′ (Figure 2).

Didehydroconicol (**2**) was isolated as a yellow oil. A molecular formula of $C_{16}H_{16}O_2$, corresponding to nine double-bond equivalents, was established by HREIMS. Except for the three methyl signals at δ 2.37 (3H, bs) and δ 1.58 (6H, s), all of the signals observed in the ^{1}H NMR spectrum of **2** in $CDCl_3$ (Table 2) were located in the region of protons attached to sp^2 carbon atoms. Two distinct spin systems could be assembled by detailed analysis of the DQF-COSY H−H experiment. The first one exhibited signals in the ^{1}H NMR spectrum reminiscent of those of a

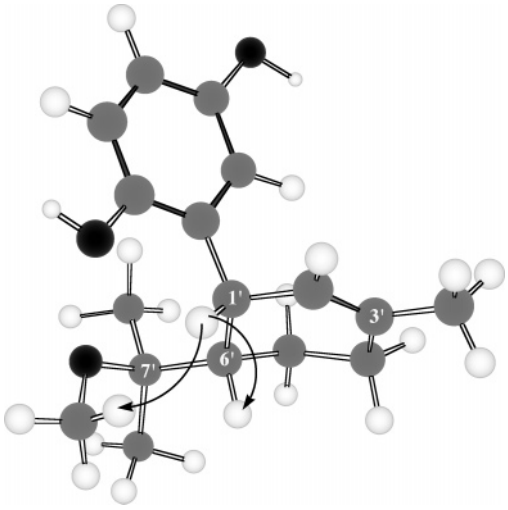

Figure 2. Proposed chemical structure of methoxyconidiol (**1**). An energy-minimized structure of **1** with the observed NOEs (arrows). Chem3D software was used to construct the model.

Table 2. ^{1}H and ^{13}C NMR Assignements of Didehydroconidiol (**2**)

atom no.	δ_C[a]	δ_H[a] (mult., *J*/Hz)	δ_H[b] (mult., *J*/Hz)	HMBC[c]
1	146.8			H-3, H-6
2	123.5			H-2, H-6
3	109.4	7.18 (d, 2.0)	7.15 (d, 2.5)	H-5
4	150.2			H-3, H-5
5	116.2	6.68 (dd, 8.0; 2.0)	6.63 (dd, 8.5; 2.5)	H-3
6	118.8	6.80 (d, 8.0)	6.71 (d, 8.5)	
1′	128.3			H-3
2′	123.0	7.43 (bs)	7.48 (d, 1.5)	H-4′, H-9′
3′	137.3			H-9′
4′	129.0	7.11 (AB)	7.11 (dd, 7.5; 1.5)	H-2′, H-9′
5′	123.2	7.09 (AB)	7.20 (d, 7.5)	
6′	137.2			H-2′, H-4′, H-5′, H8′
7′	77.4			H-8′
8′	27.5	1.58 (s)	1.49 (s)	
9′	27.5	1.58 (s)	1.49 (s)	
10′	21.3	2.37 (s)	2.33 (s)	H-2′
OH			9.03 (s)	

[a] $CDCl_3$. [b] DMSO-d_6. [c] Proton showing long-range correlation to indicated carbon.

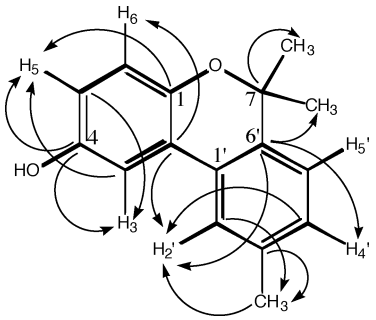

Figure 3. Long-range ^{1}H−^{13}C couplings observed for **2** from HMBC experiments (8 and 5 Hz).

2-substituted *para*-hydroquinone ring with resonances at 7.18 (H-3, d, J = 2.0 Hz), 6.80 (H-6, d, J = 8.0 Hz), and 6.68 ppm (H-5, dd, J = 8.0, 2.0 Hz) linked, respectively, to carbon atoms at 109.4, 118.8, and 116.2 ppm (HSQC cross-peaks). $^3J_{CH}$ correlations (HMBC) H-3/C-5 (δ 116.2), H-5/C-1 (δ 146.8), and H-6/C-2 (δ 123.5) completed the assignment of these aromatic ring resonances (Figure 3). Only one exchangeable proton could be observed in the ^{1}H spectrum of **2** at 9.04 ppm. By irradiation of this signal, we observed that H-3 and H-5 displayed taller and sharper

Table 3. In Vitro Biological Activities of Meroterpenes **1**, **2**, **3**, **4**, and **5** against Bacteria and Tumor Cell Lines

	E. coli MIC (mmol)	*M. luteus* MIC (mmol)	CEM-WT IC_{50} (μmol)[a]
methoxyconidiol (**1**)	>2	>2	>10 mM
didehydroconicol (**2**)	>2	0.51	>10 mM
cordiachromene A (**3**)	>2	0.51	30
epiconicol (**4**)	>2	0.13	60
conidione (**5**)	>2	>2	>10 mM

[a] 50% inhibitory concentration in a 72 h growth inhibition assay. Values shown are means of three separate experiments.

signals, indicating that the hydroxyl group is located on the vicinal carbon atom resonating at 150.2 ppm (C-4). The second aromatic system, appearing as an ABX spin system in $CDCl_3$, was formed by H-2′ at δ 7.43, H-4′ at δ 7.11, and H-5′ at δ 7.09. The use of DMSO-d_6 or CD_3OD as NMR solvent permitted a better understanding of these latter signals, exhibiting the characteristic multiplicity of three protons belonging to a 1,2,4-trisubstituted aromatic ring (Figure 3). Detailed analysis of HSQC and HMBC data allowed the assignment of this second aromatic ring system (Figure 3 and Table 2). $^3J_{CH}$ HMBC correlations H-8′/C-7′, H-8′/C-6′, and H-4′/C-6′ suggested that the two remaining equivalent methyl groups (δ 1.54, s, 6H) attached to the quaternary carbon atom C-7′ (δ 77.4) must be connected to C-6′. The aromatic methine protons at positions C-3 and C-2′ showed HMBC correlations to, respectively, carbon atoms at δ 128.3 (C-1′) and 123.5 (C-2). These key correlations defined connection between the two aromatic subunits. Since eight of the nine degrees of insaturation were attributed, compound **2** must have a third ring. The chemical shift of C-7′ led us to close the structure of didehydroconicol (**2**) by a oxygenated six-membered ring between the two aromatic moieties.

Methoxyconidiol (**1**) and didehydroconicol (**2**) together with already described cordiachromene A (**3**), epiconicol (**4**), and conidione (**5**) were evaluated for their bioactivity against bacteria and cultured cells of CEM human leukemia.

Antibacterial activity was quantitatively determined against the Gram-positive bacterium *Micrococcus luteus* and the Gram-negative bacterium *Escherichia coli* using a broth dilution method according to the NCCLS guidelines.[13] The minimum inhibition concentrations (MIC) are listed in Table 3. Compounds **1**, **2**, **3**, **4**, and **5** did not show any activity against *E. coli*. Epiconicol (MIC = 0.13 mmol), didehydroconicol (MIC = 0.52 mmol), and cordiachromene A (MIC = 0.51 mmol) showed weak activity against *M. luteus*. As bacterial growth did not restart, an aliquot of the bacteria suspension treated with cordiachromene A was placed on a solid nutritive medium; this compound caused bacterial killing and could be considered as bactericidal. On the other hand, epiconicol and didehydroconicol had an inhibitory effect on bacterial growth, thereby showing a bacteriostatic effect. Benslimane et al. had reported that cordiachromene A was bactericidal at the concentration of 128 μg/mL against *Staphylococcus aureus* and at 64 μg/mL against S*treptococcus faecalis*.[4]

Growth-inhibitory effects of meroterpenes were also evaluated on a sensitive human tumor cell line. The IC_{50} (Table 3) values were determined for drug-sensitive CCRF-CEM human leukemic lymphoblastics cells. Methoxyconidiol (**1**), didehydroconicol (**2**), and conidione (**5**) did not show activity in the range of concentrations used (10^{-4} to 1 mg/mL). Cordiachromene A (**3**) and epiconicol (**4**) caused dose-dependent decreases in cell proliferation. Cordiachromene A had an IC_{50} value of 30 μmol and epiconicol 60 μmol. The moderate cytotoxicity of compound **4** can be compared to the result obtained by Carroll et al. (IC_{50} 10 μg/mL) on a panel of cultured cell lines (P388, A549, HT29, CV1).[9]

Experimental Section

General Experimental Procedures. IR and UV spectra were recorded, respectively, on a Perkin-Elmer 1600 FTIR and a Perkin-Elmer 551 spectrometer. Nuclear magnetic resonance (NMR) spectra were recorded on a JEOL EX 400 spectrometer with the solvent chloroform ($CDCl_3$) used as an internal standard. High-performance liquid chromatography (HPLC) was performed on an Interchrom Uptisphere-5μm-ODB column with Jasco 880-PU pumps, 7125 Rheodyne injectors, and either a Polymer Laboratories evaporative light scattering detector ELS 1000 or a Waters 996 photodiode array detector. Vacuum column chromatography was performed on silica gel 60 Merck (0.063−0.200 mm).

Animal Material. Specimens of *Aplidium aff. densum* (Giard, 1872) (Polyclinidae) were collected by hand using scuba at Masirah Island (Oman) and preserved in ethanol. Identification was performed by Dr. X. Turon.

Extraction and Isolation Procedures. The ascidian preserved in ethanol was exhaustively extracted three times with EtOH, then a mixture of EtOH/CH_2Cl_2, and finally CH_2Cl_2 to give the total crude extract (156.3 g) after evaporation of the solvents under vacuum. Partition of this residue between water and ethyl acetate provided 28.6 g of organic-soluble material, which was first chromatographed on a silica column using stepwise elutions with heptane and ethyl acetate. The fraction eluted with heptane/ethyl acetate (80/20) (490 mg) was subjected to silica flash column chromatography using the same mixture as the eluant to provide the terpenic fraction (125 mg). This fraction was further purified by RP-18 HPLC using isocratic conditions, MeOH/H_2O, 85:15 (v/v), at a flow rate of 3 mL/min, to afford cordiachromene A (**3**, 32 mg), epiconicol (**4**, 15 mg), conidione (**5**, 13 mg), methoxyconidiol (**1**, 9 mg), and didehydroconicol (**2**, 14 mg).

NMR Measurement Conditions. All spectra were obtained with a NM-40TH5 dual ^1H, ^{13}C probe in a JEOL EX400 operating at 400 MHz for proton and 100.53 MHz for carbon-13 at 298 K. ^1H and ^{13}C NMR chemical shifts are referenced to solvent peaks: δ_H 7.24 (residual $CHCl_3$), δ_C 77.0 for $CDCl_3$. Methoxyconidiol (5 mg) and didehydroconidiol (5 mg) were dissolved in a 5 mm tube in 0.75 mL of $CDCl_3$. Two-dimensional (2D) homonuclear correlated experiments DQF-COSY were acquired using standard procedures with a spectral width of ca. 4000 Hz in both columns F1 and F2. Heteronuclear correlated experiments were performed in ^1H-detected mode using the standard pulse programs HSQC and HMBC with a spectral width of ca. 20 000 Hz in F1 and 4000 Hz in F2. The evolution delay was set to optimize 140 Hz couplings for HSQC and 8 and 5 Hz couplings for HMBC.

Methoxyconidiol (1): yellow oil; $[\alpha]^{23}_D$ +6.4° (c 2 × 10^{-5}, $CHCl_3$); UV (MeOH) λ_{max} (log ϵ) 228 (3.7623), 274 (3.3368), 296 (3.4581) nm; IR ($CHCl_3$) ν_{max} 3319, 1608, 1491, 1458 cm^{-1}; ^1H and ^{13}C NMR, see Table 1; EIMS *m/z* 276 $[M]^+$ (7), 244 $[M - OCH_3]^+$ (25), 225 $[M - OCH_3 - H_2O]^+$ (21), 201 (27), 161 (52), 73 (100); HREIMS *m/z* 276.1734 (calcd for $C_{17}H_{24}O_3$, 276.1725).

Didehydroconidiol (2): yellow oil; UV (MeOH) λ_{max} (log ϵ) 230 (3.5866), 240 (3.4610), 254 (3.2931), 272 (3.2659), 330 (2.9978) nm; IR ($CHCl_3$) ν_{max} 3599, 3309, 1602, 1502, 1457 cm^{-1}; ^1H and ^{13}C NMR, see Table 2; EIMS *m/z* 240 (17), 225 (100); HREIMS *m/z* 240.1155 (calcd for $C_{16}H_{16}O_2$ 240.1150).

Antibacterial Assay. The technique used was based on a method published by the National Committee of Laboratory Safety and Standards (NCLSS) in 1997.[12] Products dissolved in DMSO (not exceeding 5%, total volume) were incubated with two bacterial strains (Institut Pasteur, Paris), a Gram-positive (*Micrococcus luteus*) and a Gram-negative (*Escherichia coli*), in 96-well plates (MERCK) in PB medium, at 37 °C during 24 h, under stirring. Assays were carried out in triplicate and the results averaged. Growth was evaluated by reading optical

density (630 nm). When an activity was detected (absence of growth), a sample of media was taken on rich solid medium (Petri dishes) to establish the effect (bacteriostatical or bactericidal effect).

Antiproliferative Tests. The acute lymphoblastic leukemia CEM-WT cells were obtained from Dr. W. T. Beck, St. Jude Children's Research Hospital, Memphis, TN. The cell line was grown in 25 mL plastic tissue culture flasks using RPMI 1640 medium supplemented with 2 mmol of l-glutamine (ICN, Orsay, France), 10% fetal calf serum (v/v) (Gibco, Eragny, France), and 1% of antibiotics solution (penicillin and streptomycin) in a humidified atmosphere of 5% carbon dioxide in air at 37 °C. Serial dilutions of compounds were prepared in the culture medium. The drug at the appropriate concentration was added to cell cultures (2×10^5 cells/mL) for 2 days without renewal of the medium. Cells were then enumerated using a Beckman Z1 particle counter. A counting was made at 4 h, then at 8, 24, 32, 48, 56, and 72 h, then each 24 h (to 120 h of incubation maximum). Assays were carried out in triplicate and the results averaged. The concentration of drugs required to inhibit growth of cells by 50% (IC_{50}) in 72 h was determined.

Acknowledgment. We thank the Ardoukoba Association for help in collecting the Oman samples of *Aplidium aff. densum*. We are grateful to X. Turon for identification of the ascidian and to A. Al Mourabit for $[\alpha]_D$ measurement. Financial support for this research was provided by La Ligue Contre le Cancer (Comité des Pyrénées-Orientales).

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NP050110P