Cytotoxic Diterpenoids from the Brown Alga Dilophus ligulatus

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CYTOTOXIC DITERPENOIDS FROM THE BROWN ALGA *DILOPHUS LIGULATUS*

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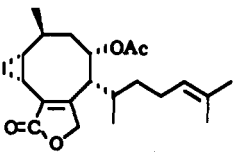
ABSTRACT.—A new xenicane-type diterpene, dilopholide [**5**], and four known diterpenoids, acetoxycrenulide [**1**], acetylcoriacenone [**2**], and its epimer isoacetylcoriacenone [**3**], and hydroxyacetyldictyolal [**4**], have been isolated from the brown alga *Dilophus ligulatus* [syn. *spiralis* (Montagne) Hamel]. The structure of dilopholide [**5**] was elucidated by spectroscopic methods, including the concerted application of a number of 2D nmr techniques, including ^{1}H-^{1}H COSY, heteronuclear proton-carbon chemical shift correlation (HETCOSY), and long-range HETCOSY. The cytotoxic activity of compounds **1–5** has been examined against several types of mammalian cells: human nasopharynx carcinoma cells (KB), human lung carcinoma cells (NSCLC-N6), murine leukemia cells (P-388), and murine leukemia cells expressing the multi-drug-resistance gene, mdr (P-388/DOX).

Brown algae of the family Dictyotaceae, particularly species belonging to the closely related genera *Dictyota* and *Dilophus*, are known as prolific producers of biologically active secondary metabolites (1). We have recently examined the crude extract of *Dilophus ligulatus* (Kütz) Feldm. [syn. *Dilophus spiralis* (Montagne) Hamel], which had shown antimicrobial activity in a previous screening of Mediterranean Dictyotaceae (2). This led to the identification of nine bioactive diterpenoids, all previously known from other marine sources (3).

A more recent fractionation of this extract afforded five additional metabolites **1–5**, displaying cytotoxic activity to several types of mammalian cells in culture (KB, P-388, P-388/DOX, and NSCLC-N6). A comparison of the spectral properties (ms, ir, ^{1}H- and ^{13}C-nmr) of these compounds with literature data led to the identification of acetoxycrenulide [**1**], previously known from *Dictyota crenulata* (4), acetylcoriacenone [**2**] and its epimer isoacetylcoriacenone [**3**], found in *Pachydictyon coriaceum* (5), and hydroxyacetyldictyolal [**4**], known from *Dictyota dichotoma* (6). The novel xenicane diterpenoid dilopholide [**5**] was also obtained, and we report here its structural establishment, complete ^{1}H- and ^{13}C-nmr assignment, and a conformational study based on nOe data. The biological properties of compounds **1–5** are also reported.

RESULTS AND DISCUSSION

Dilopholide [**5**] (0.002% dry wt) was purified as a colorless oil, $[\alpha]_D$ −113.7° from the lipid extract of *Dil. ligulatus*. The molecular formula $C_{22}H_{32}O_4$ was established by hreims (observed 360.2297, required 360.2300). Its ir spectrum revealed a carbonyl absorption at 1760 cm^{-1}. The presence of an acetate ester was inferred from the eims peaks at *m/z* 318 (*m/z* 318.2193, calcd 318.2195 $[M-COCH_2]^+$) and *m/z* 300 $[M-HOAc]^+$. The ^{13}C-nmr spectrum of **5** exhibited the expected 22 carbon resonances (Table 1) assigned by a DEPT spectrum to five quaternary carbons, seven methine carbons, five methylenes, and five methyl groups. A closer inspection of the ^{13}C-nmr spectrum confirmed the presence of carbonyl (178.2 ppm) and acetate (166.8, 20.71 ppm) groups and showed six olefinic carbon resonances (122.7, 123.8, 127.6, 130.4, 132.1, and 134.7 ppm). These data suggested that **5**, having seven degrees of unsaturation, could be a bicyclic diterpene derivative.



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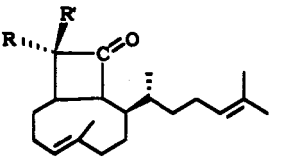
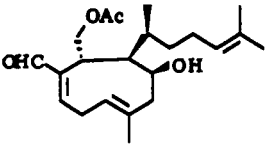


2 R=OAc, R′=H
3 R=H, R′=OAc



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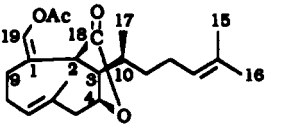

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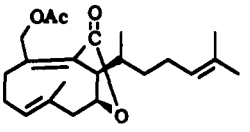

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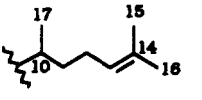
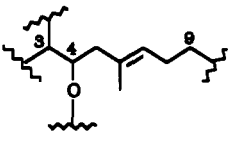


A



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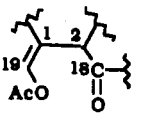

C

A heteronuclear 2D nmr experiment (proton-carbon chemical shift correlation, HETCOSY) allowed the correlation of all proton resonances with their corresponding carbon resonances, as reported in Table 1. Only one oxygenated sp^3 carbon (methine) could be inferred from the ^{13}C-nmr (79.4 ppm) and ^{1}H-nmr (4.55 ppm) resonances. The fragments at *m/z* 109 and 69 in the eims spectrum suggested the presence of a 6-methyl-5-hepten-2-yl side chain **A**, typical of the Dictyotaceae diterpenoids. The nmr resonances attributable to positions from C-10 to C-17 fit well with literature data (7).

To elucidate the structure of this algal metabolite, a spectral analysis based on the combined use of 2D nmr techniques (proton-proton and proton-carbon chemical shift correlations: COSY, one-bond HETCOSY, and long-range HETCOSY, as reported in Table 1) was carried out.

The oxygen-bearing methine signal in the ^{1}H-nmr spectrum of **5** (4.55 ppm, H-4) was a convenient starting point for the analysis. The COSY spectrum showed correlations of this signal with three further protons at 1.89, 2.23, and 2.70 ppm. The 1.89 and

TABLE 1. Nmr Spectral Data for Compound **5**.

Position	^{13}C shifts[a]	^{1}H shifts[b]	HETCOSY[c]	COSY[d]	NOESY[e]
1	122.7 (s)		H-2, H-8α, H-9α		
2	48.0 (d)	3.22 (t, 2.5)		H-3, H-19	H-3, H-10, H_3-17
3	48.7 (d)	2.23 (m)	H-4, H-10, H_3-17	H-2, H-4, H-10, H_3-17	H-2, H-4, H_2-11, H_3-20
4	79.4 (d)	4.55 (dt, 4, 1.8)	H-5α	H-3, H-5α	H-3, H-5α, H-5β, H_2-11, H_3-17
5α	41.4 (t)	2.70 (brd, 15)	H-3, H_3-20	H-4, H-5β	H-4, H-5β, H-7
5β		1.89 (dd, 15, 4)		H-4, H-5α	H-4, H-5α, H_3-20
6	130.4 (s)		H-4, H-5α, H-5β, H_3-20		
7	127.6 (d)	5.37 (ddq, 12.0, 5.0, 1.5)	H-5α, H-9α, H_3-20	H-8α, H-8β, H_3-20	H-5α, H-8α, H_3-22
8α	29.3 (t)	2.58 (m)		H-7, H-8β, H-9α, H-9β	H-7, H-8β, H-9α
8β		2.27 (m)		H-7, H-8α, H-9α, H-9β	H-8α, H-9β, H_3-20
9α	30.0 (t)	2.23 (m)	H-8α	H-8α, H-8β, H-9β	H-8α, H-19
9β		2.10 (m)		H-8a, H-8β, H-9α	H-8β
10	36.7 (d)	1.98 (m)	H-2, H_2-11, H_3-17	H-3	H-2, H_2-11, H_3-17
11	35.3 (t)	1.27 (m)	H-10, H_2-12, H_3-17	H-3, H_2-11, H_3-17	H-3, H-4, H-10, H_2-12, H_3-17
12	26.0 (t)	1.98 (m)	H_2-11	H_2-11, H-13	H_2-11
13	123.8 (d)	5.06 (brt, 7)	H_2-11, H_2-12, H_3-15, H_3-16	H_2-12, H_3-15, H_3-16	H_3-15
14	132.1 (s)		H_3-15, H_3-16		
15	25.7 (q)	1.64 (brs)	H_2-11, H-13, H_3-16	H-13, H_3-16	H-13
16	17.6 (q)	1.57 (brs)	H_3-15	H-13, H_3-15	
17	13.4 (q)	0.87 (d, 7)	H-3, H_2-11	H-3, H_2-11	H-2, H-4, H-10, H_2-11
18	178.2 (q)		H-2, H-4		
19	134.7 (d)	7.04 (d, 2.5)	H-2	H-2	H-9α, H_3-22
20	21.8 (q)	1.70 (d, 1.5)	H-5α	H-7	H-3, H-5β, H-8β
21	166.8 (s)		H-19, H_3-22		
22	20.7 (q)	2.11 (s)			H-7, H-19

[a]Recorded at 75.47 MHz in $CDCl_3$. Multiplicities were determined by a DEPT experiment and are reported in parentheses.
[b]Recorded at 300 MHz in $CDCl_3$. Multiplicities and *J* values (Hz) reported in parentheses.
[c]Long-range carbon-proton correlations observed in the long-range HETCOSY spectrum.
[d]Proton-proton scalar correlations observed in the COSY spectrum.
[e]Proton-proton dipolar correlations observed in the NOESY spectrum.

2.70 protons were geminally coupled, as confirmed by the one-bond HETCOSY experiment, fixing also the related carbon resonance (41.4 ppm, C-5). These methylene protons did not show any further coupling, and consequently must be adjacent to a quaternary carbon. This could be assigned at 130.4 ppm (C-6) on the basis of the long-range HETCOSY experiment, showing cross-correlations of C-6 with H-4 and H_2-5. Further heteronuclear couplings were observed between C-5 and the vinyl methyl resonating at 1.70 ppm, in turn one-bond-correlated with the carbon signal at 21.8 ppm (C-20). Reciprocally, this carbon showed heteronuclear correlation with the methylene proton at 2.70 ppm. Further inspection of the COSY spectrum allowed expansion of this partial structure. The 1.70 ppm signal showed long-range correlation with the olefin resonance at 5.37 ppm (H-7), in turn coupled with two signals at 2.27 and 2.58 ppm (H_2-8), due to unequivalent methylene protons (COSY, one-bond HETCOSY). These were further correlated to a 2H multiplet resonating at 2.10 and 2.23 ppm, which were also mutually coupled (COSY). From one-bond HETCOSY, it appeared clearly that two different proton signals overlapped at 2.23 ppm. These were related to a methylene (30.0 ppm, C-9) and a methine (48.7 ppm, C-3) carbon, the latter being connected (long-range HETCOSY) to the above-cited signal at 4.55 ppm (H-4). Thus the partial structure **B**, connecting positions 3 to 9, was determined.

A low-field proton resonance at 3.22 ppm, one-bond connected to the methine carbon at 48.0 (C-2), showed long-range correlation (COSY) to the signal at 7.04 ppm, in turn one-bond connected to the sp^2 carbon resonating at 134.7 ppm (C-19). The long-range HETCOSY spectrum showed cross-correlations of H-2 with the carbon signals at 178.2 (C-18), 134.7 and 122.7 (C-1); in addition, H-19 exhibited a long-range correlation to the carbonyl peak at 166.8 ppm, which was in turn correlated to the acetate proton signal at 2.11 ppm. These data could be assembled in the partial structure **C**, including an enol acetate function. The ir (1760 cm^{-1}) and uv (λ max 217, ϵ 5624) absorptions and low-field chemical shift of H-19 supported this hypothesis.

Partial structure **A** was firmly established by use of COSY and HETCOSY correlations, as reported in Table 1. Joining of partial structures **A** and **B** was based on the long-range HETCOSY spectrum. Indeed, the carbon signal at 48.7 ppm (C-3) showed ^2J C,H correlation with the proton signal at 1.98 ppm (H-10, one-bond-connected to the carbon at 36.7 ppm); conversely, the C-17 methyl at 13.4 ppm was ^3J heterocorrelated with the H-3 signal. Due to overlapping of H-9α and H-3 resonances, joining of partial structures **B** and **C** could not be based on the cross-correlations involving the 2.23 ppm signal; nevertheless, a ^3J heterocorrelation between C-10 and H-2 allowed the establishment of the C-2/C-3 connection, and analogously the observed cross-correlation between C-1 and H-8α led to establishment of the C-1/C-9 connection. Consequently, a lactone ring closure between C-4 and C-18 is required; this is supported by the correlation (long-range HETCOSY) between C-18 and H-4 and by the ir carbonyl absorbance (1760 cm^{-1}), overlapped with that of the enol acetate. Once the complete carbon framework of dilopholide [**5**] had been determined, it became evident that this metabolite belongs to the class of xenicane diterpenoids, a well-known group of marine metabolites, including dictyotalide B [**6**] (8), an isomer of dilopholide identified also in our previous investigation of *Dil. ligulatus* extract (3).

A stereochemical study of dilopholide has been carried out, largely by the use of NOESY (Table 1). The *E* configuration has been assigned to the C-6 double bond based on the observed nOe correlation between H-7 and H-5α (Table 1). Lack of nOe correlations between H-7 and Me-20 and the consideration that a trans configuration at C-6 is widespread among xenicane diterpenoids give further support to this assumption. The configuration at C-1 has been defined as *Z* on the basis of the nOe correlations between H-19 and H-9α. The relative stereochemistry at the chiral centers of the

molecule has been assigned on the basis of following considerations. Inspection of Dreiding stereomodels showed that a lactone ring closure without undue steric hindrance requires the relative stereochemistry at C-2 (*R**) and C-4 (*S**) be assigned to dilopholide, the same previously reported for xenicane lactones (8). This is in agreement with the nOe correlations observed for H-2 and H-4 (see Table 1), proving their close proximity to the side chain **A**. The relative stereochemistry at C-3 (*S**) has been fixed on the basis of the measured J values for H-2 and H-4, which allow indirect estimation of $J_{2,3}$ (2.5 Hz) and $J_{3,4}$ (1.8 Hz). Both of these values require a cis relationship between H-3 and either H-2 or H-4. This is also in agreement with the observed nOe correlations between H-3 and Me-20. Finally, configuration at C-10 (*R**) has been proposed on the basis of biogenetic considerations, and it is by far the most commonly reported configuration for Dictyotaceae diterpenes possessing the side chain **A** (9,10). The preferred conformation in solution of dilopholide [**5**] has been determined on the basis of NOESY data, as reported in Figure 1. The nine-membered ring adopts a 'crown' conformation, as expected from previous X-ray and conformational studies of xenicanes (8–12); the conformation determined for **5** appears slightly different with respect to those reported for other xenicane lactones (8).

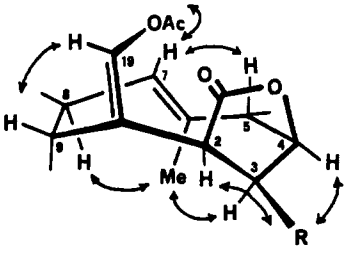

FIGURE 1. Preferred conformation of dilopholide [**5**] based on nOe data (↦nOe).

The complete ^{1}H- and ^{13}C-nmr assignments for dilopholide [**5**], based on the 2D nmr experiments discussed above, are reported in Table 1.

The results of cytotoxicity bioassays for compounds **1–5** are reported in Table 2. Dilopholide [**5**] showed significant cytotoxic activity (ED_{50}<4 μg/ml) against KB (human nasopharynx carcinoma) cells, NSCLC-N6 (human lung carcinoma) cells, and P-388 (murine leukemia) cells.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—Nmr spectra were recorded at 300 MHz for ^{1}H and 75 MHz for ^{13}C on a Bruker AC300 TF spectrometer. All chemical shifts are reported as ppm with respect to TMS

TABLE 2. Cytotoxicities of Compounds **1–5** (ED_{50}, μg/ml).

Compound	Cell line			
	KB	P-388	P-388/DOX	NSCLC-N6
1	>10	>10	>10	>10
2	>10	4.40	>10	5.40
3	>10	6.00	>10	6.82
4	2.10	3.90	9.30	10.00
5	1.50	0.50	12.00	3.30
Mercaptopurine[a]	0.54	0.70	0.25	0.76

[a]Positive standard control.

(δ=0). The ir spectrum (KBr) was recorded on a Nicolet 20SxC spectrophotometer. The mass spectrum was determined on a VG-ZAB-SEQ mass spectrometer. The uv spectrum was recorded on a Perkin-Elmer Coleman 571 spectrophotometer.

PLANT MATERIAL.—*Dil. ligulatus* was collected by scuba diving in the bay of Villefranche/Mer (France) in May 1990. The plant material was identified by Dr. B. Caram, Laboratoire de Protistologie Marine, Station Marine de Villefranche/Mer (France). The voucher specimen was deposited at the Algal collection, INSERM U303, Villefranche sur Mer, France.

BIOASSAYS.—The cytotoxicity tests against KB cells, P-388 cells, P-388/DOX, and NSCLC-N6 cells were performed in the S.M.A.B., groupe de recherche sur les Substances Marines à Activité Biologique, U.E.R. des Sciences Pharmaceutiques, Université de Nantes (France), using standard protocols with mercaptopurine as a positive standard.

EXTRACTION AND ISOLATION.—Air-dried alga (644 g) was ground and extracted with EtOH (2×3 liters) with stirring. After solvent removal, the residue (18 g) was taken up in hexane. The hexane extract was chromatographed on Si gel (40–60 μm) eluted with increasing amounts of EtOAc in hexane. The fraction eluted with 20% EtOAc was evaporated and chromatographed on reversed-phase preparative hplc (300×25 mm, C18, 12–40 μm, C.E.D.I., 20% H_2O/MeOH). Complete purification of the active fraction was achieved by hplc (250×10 mm, Si gel 10 μm, Interchrom IS10.25F, 20% EtOAc/hexane) which gave compounds **1** (0.0040% alga dry wt), **2** (0.0015% alga dry wt), **3** (0.0020% alga dry wt), **4** (0.0030% alga dry wt), and **5** (0.0020% alga dry wt).

Dilopholide [**5**].—Colorless oil: $[\alpha]_D$ −113.7° (c=0.86, $CHCl_3$) (measured on a Beckmann DB-GT polarimeter); uv (MeOH), λ max 217 nm (ϵ=5624); ir (KBr) ν max 2962, 2927, 2876, 1760, 1660, 1453, 1384, 1212, 1179, 1115, 1074, 1023, 999, 945, 907, 865 cm^{-1}; ^1H and ^{13}C nmr see Table 1; eims *m/z* $[M]^+$ 360.2297 ($C_{22}H_{32}O_4$, calcd 360.2300), 318 (4%), 300 (4%), 231 (5), 161 (18), 135 (20), 109 (27), 91 (15), 82 (28), 77 (11), 69 (83), 43 (83), 41 (100), 28 (100).

ACKNOWLEDGMENTS

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